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PTEROYLGLUTAMIC ACID DEFICIENCY IN SWINE; EFFECTS OF TREATMENT WITH PTEROYLGLUTAMIC ACID, LIVER EXTRACT, AND PROTEIN

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THE discovery of the effectiveness of pteroylglutamic acid in the treatment of the macrocytic anemias has, so far, failed to clarify our understanding of the pathogenesis of these anemias.[1] It has now been demonstrated that neither pteroylglutamic acid nor its naturally occurring conjugate, the heptaglutamate, is the extrinsic factor, the intrinsic factor, or the potent antipernicious anemia substance in purified liver extract. Furthermore, it has become increasingly evident that pteroylglutamic acid neither corrects nor prevents the neurological manifestations of pernicious anemia.[2] With regard to the role of pteroylglutamic acid in pernicious anemia, it has been suggested that in some patients with this disorder there is, in addition to other metabolic defects, an inability to utilize the naturally occurring pteroylheptaglutamate.[1] It has also been proposed that pteroylglutamic acid and the antipernicious anemia principle of liver may function via unrelated pathways.[1]

The purpose of this paper is to describe the hematologic manifestations of pteroylglutamic acid deficiency in swine produced by the administration of a synthetic substance having an action antagonistic to that of pteroylglutamic acid and to compare the therapeutic effectiveness of purified liver extract and synthetic pteroylglutamic acid in this condition. This report leaves many questions unanswered and therefore must be considered preliminary in nature. The observations are now being amplified and extended.

The pteroylglutamic acid antagonist used in these experiments (N67†) was prepared‡ by allowing 2, 4, 5-triamino-6-hydroxypyrimidine and p-aminobenzoyl-l(+)-glutamic acid to react with 2, 3-dibromobutyraldehyde. Since the reaction product (N67) has not been purified, it has been termed crude methylfolic acid. The product of the reaction described has been designated by Martin, Tolman and Moss[3] as 7-methylfolic acid. The substance antagonizes reversibly the effect of pteroylglutamic acid on the growth of *Streptococcus faecalis R* and of *Lactobacillus casei*.[4] Different inhibiting ratios have been reported for the two organisms. The ratio of antagonist to pteroylglutamic acid for *Str. faecalis R* is 20:1 and for *L. casei* about 1,000:1.

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Studies in chicks,[3] mice,[5] rats,[4] and swine[6, 7] have been reported in which the antagonist described has been used. In rats the syndrome produced was similar to, but somewhat more severe than, that produced by the feeding of a purified diet plus succinylsulfathiazole, namely a slowing of growth, inanition, anemia, marked leucopenia and neutropenia. Bone marrow studies revealed an increase in the erythroid cells, especially in the more immature ones, and a diminution in mature granulocytes, thus indicating that maturation of both erythroid and myeloid cells was seriously impaired. The animals usually became moribund within one to two weeks after the onset of the syndrome, at which time they were extremely emaciated. At autopsy, necrotic and ulcerative lesions were found in the oral cavity. The syndrome could be reversed completely by the administration of synthetic pteroylglutamic acid, the inhibiting ratio of antagonist to pteroylglutamic acid being about 3,000:1. Reversal of the syndrome was accompanied by temporarily overcompensatory increases in the total leucocyte and granulocyte counts. Enlargement of the spleen was noted following recovery.

Welch, Heinle, and colleagues[6, 7] have reported the production of macrocytic anemia with megaloblastic hyperplasia of the bone marrow in three pigs maintained on a highly purified diet essentially free of extrinsic factor to which succinylsulfathiazole and the antagonist mentioned previously were added. One of the animals responded to the administration of crude sodium caseinate together with a 95 per cent ethanol extract of crude casein and normal human gastric juice. This animal later relapsed and was treated successfully with a single injection of 15 units of liver extract. The second animal responded rapidly to four daily intramuscular injections of 10 mg. of pteroylglutamic acid. The third pig became critically ill and was successfully treated with a combination of purified liver extract, pteroylglutamic acid, and niacinamide. Their conclusion that purified liver extract is effective in correcting the deficiency of pteroylglutamic acid in pigs[7] is in contrast to the experimental evidence in other species. Chicks,[8] rats,[8] and monkeys[9] deficient in pteroylglutamic acid do not respond to purified liver extracts.

EXPERIMENTAL PROCEDURE

For this study twelve weanling Chester-White pigs, 21 to 28 days of age, were used. Eight animals served as controls and four were given the crude methylfolic acid antagonist (N67) as well as sulfasuxidine. Pteroylglutamic acid, p-aminobenzoic acid, and inositol were omitted from the vitamin supplement of the pigs receiving antagonist.

The basal diet consisted of Sheffields' New Process casein, 10 per cent, sucrose, 57.7 per cent, lard, 27.1 per cent, and swine salt mixture,[10] 5.2 per cent. This was fed in amounts of 29.9 Gm. (152 calories) per kilogram body weight per pig per day. In addition, all animals received Natola,* 0.056 Gm. per kilogram body weight per week. Vitamins were supplied in crystalline form by placing them in capsules and administering them orally three times a week. The quantities of crystalline vitamins, when these were given, were as follows

*Parke, Davis & Company, Detroit, Mich.; 55,000 units of vitamins A, 11,000 units of vitamin D per gram.

(milligrams per kilogram body weight daily): thiamin hydrochloride, 0.25; riboflavin, 0.12; nicotinic acid, 1.20; pyridoxine hydrochloride, 0.20; calcium pantothenate, 0.50; choline chloride, 50.00; para-aminobenzoic acid, 0.10; inositol, 0.10; pteroylglutamic acid, 0.30. In addition, all animals received crystalline biotin, 50 μg per kilogram body weight per week intramuscularly. Sulfasuxidine, when given, was added to the basal diet in amounts of 2.0 per cent. The antagonist, when given, was administered daily in capsules in amounts of 0.2 per cent of the basal diet (equivalent to 0.06 Gm. per kilogram body weight). Full details of the experimental methods have been published elsewhere.[10]

The four animals receiving the antagonist were first depleted of pteroylglutamic acid for periods of eighty-six to one hundred twenty-two days by a diet lacking pteroylglutamic acid, supplemented with sulfasuxidine.

Plasma iron determinations were made by the method of Barkan and Walker.[11] Serum copper was measured by the method of Cartwright, Jones, and Wintrobe.[12] For the determination of free erythrocyte protoporphyrin, the method of Grinstein and Watson[13] was used.

Specimens of bone marrow were obtained by aspiration of the sternal marrow with standard 16 gauge sternal puncture needles. A small amount of marrow fluid, usually less than 0.3 ml., was withdrawn into a clear dry syringe, and thin cover-glass preparations were drawn and stained with Wright's stain. From 500 to 1,500 cells were counted in each preparation. The figures are expressed in per cent of total cells counted.

In order to determine whether or not extrinsic factor is present in the crude casein used in these studies, the material was assayed in three patients with classic, uncomplicated addisonian pernicious anemia. The procedure of assay was as follows. The patients were hospitalized and during the assay periods liver, meat, meat products, milk, and poultry were excluded from the diet. Bread, cereals, sugar, fats, vegetables, and fruits were permitted in the amounts desired. Fifty grams of the casein to be assayed were incubated at 37° C. for two hours with 150 to 200 ml. of normal human gastric juice at pH 2.5 to 3.5. The incubation mixture was then strained through cheesecloth; the filtrate was neutralized to pH 5 and administered immediately to the patient. The results

TABLE I. ASSAY OF CRUDE CASEIN FOR EXTRINSIC FACTOR ACTIVITY

PATIENT	PERIOD		THERAPY		MAX. RETICS. (%)	VOLUME PACKED RED CELLS (C.C./100 C.C.)	
	No.	DURATION (DAYS)	TYPE	DURATION (DAYS)		BEGIN.	END
J. S.	I	8	None	8	1.0	27.8	24.2
	II	20	Crude casein + alcoholic extract	10	8.4	24.2	34.0
	III	12	Liver extract, 1 u/day I.M.	10	1.8	34.0	39.0
N. B.	I	12	None	12	4.4	23.4	23.8
	II	12	Extracted casein + 3rd alcoholic extract	10	12.8	23.8	29.0
	III	10	Liver extract, 1 u/day I.M.	10	2.6	29.0	33.8
S. S.	I	22	None	22	2.2	26.4	27.0
	II	16	Crude casein	10	9.0	27.0	31.8
	III	10	Liver extract, 1 u/day I.M.	10	0.8	31.8	32.0

of the assays are presented in Table I. Patient J. S. was given, in addition to the filtrate, an alcoholic extract from crude casein. This was prepared by adding 18 liters of 65 per cent ethanol to 6 kg. of casein. The casein-alcohol mixture was allowed to stand for forty-eight hours at room temperature with intermittent shaking. The alcohol was then filtered off and distilled in vacuo to dryness. The residue was extracted with several volumes of ethyl ether to remove the fats, air dried at room temperature, and then pulverized. One-tenth of the resulting residue was added daily to the crude casein prior to incubation.

As seen in Table I, following the administration of the described mixture to Patient J. S. a reticulocytosis of 8.4 per cent developed and in twenty days the volume of packed red cells increased from 24.2 ml. per 100 ml. to 34.0. One unit of purified liver extract (15 units per milliliter) was then administered intramuscularly daily for ten days. No secondary reticulocyte response occurred. Patient N. B. was given crude casein, extracted three times as described, plus the residue from the third alcohol extraction. As can be seen, the extraction procedure failed to remove the extrinsic factor activity and a reticulocytosis developed which reached a maximum of 12.8 per cent. One unit of liver extract daily again failed to elicit a secondary reticulocytosis. Patient S. S. was given 50 Gm. of the crude casein daily without the addition of an alcoholic extract. A reticulocytosis of 9.0 per cent developed and the volume of packed red cells rose from 27.0 ml. per 100 ml. to 31.8 in sixteen days. Following this, the daily intramuscular injection of 1 unit of purified liver extract failed to produce a secondary reticulocytosis.

Since the administration of a total of 500 Gm. of crude casein caused a significant response in Patient S. S. and since the animals received approximately 15 to 120 Gm. of crude casein daily for about 190 days, it must be concluded that our experimental animals were receiving substantial amounts of extrinsic factor. It is unlikely that the response in the patients with pernicious anemia was due to the pteroylglutamic acid content of the crude casein since the total pteroylglutamic acid content of the casein, after enzymatic digestion, as determined titrimetrically with *L. casei* by the method of Teply and Elvehjem,[14] was found to be only 0.009 μg per 100 Gm. of casein. The alcohol extracted casein was found to contain only 0.006 μg per cent by this method.*

RESULTS

General.—The animals receiving the pteroylglutamic acid antagonist became listless and weak and ate poorly. Hair loss was not extensive but the hair became thin and lusterless. The general appearance of the animals was extremely untidy. The abdominal walls of the pigs sagged, probably due to loss of muscle tone. As the deficiency progressed, their squeals became faint and weak.

A moderately severe diarrhea was present, the stools being somewhat orange-yellow in color due presumably to the presence of antagonist. Growth was poor, and about equal, in both the control and antagonist groups due to the low content of protein (10 per cent) in the diet. Oral lesions were not observed.

*We are indebted to Dr. J. M. Cooperman, Hoffmann-La Roche, Inc., Nutley, N. J., for these determinations.

Peripheral Blood.—In contrast to the control animals, those receiving the antagonist became markedly anemic in twenty-one to forty-two days. The volume of packed red cells decreased from a level of about 35 ml. to approximately 20 ml. per 100 ml. (Table II, Figs. 1-4). The anemia at this time was normocytic. Examination of the blood smears revealed marked anisocytosis without a significant degree of poikilocytosis, an increase in Howell-Jolly bodies, frequent nucleated red blood cells, and moderate polychromatophilia. Unusually large macrocytes were frequently seen but microcytes were equally numerous. This is illustrated in Fig. 6.

TABLE II. DATA ON ANEMIA AND RED BLOOD CELL MORPHOLOGY IN LOW PROTEIN CONTROL GROUP AND PGA ANTAGONIST GROUP

	LOW PROTEIN CONTROL					MEAN	PGA ANTAGONIST				MEAN
Pig	10-45	10-46	10-47	10-48	10-50		10-53	10-54	10-56	10-64	
Days on experiment	156	156	130	156	156	151	164	113	105	146	132
Days on antagonist	0	0	0	0	0	0	42	25	21	36	31
R.B.C. $\times 10^6$ (c.mm.)	6.96	6.36	5.59	6.35	6.51	6.35	3.53	3.16	2.76	4.03	3.37
Hb. (Gm. %)	12.0	11.8	10.3	12.0	11.3	11.5	8.1	5.7	5.6	6.7	6.5
Ht. (c.c./100 c.c.)	36.0	35.2	30.0	36.4	34.4	34.4	24.4	18.0	15.0	20.8	19.5
M.C.V. (c.μ)	52	55	54	57	53	54	69	57	54	52	58
M.C.H. ($\gamma\gamma$)	17	19	18	19	17	18	23	18	18	17	19
M.C.H.C. (%)	33	33	34	33	33	33	35	32	34	32	33
Retics. (%)	1.0	2.6	2.0	2.0	0.6	1.6	4.0	4.4	2.6	1.2	3.0

Ht., Volume of packed red cells; M.C.V., mean corpuscular volume; M.C.H., mean corpuscular hemoglobin; M.C.H.C., mean corpuscular hemoglobin concentration.

As shown in Table III, the animals receiving the antagonist developed a mild leucopenia and severe neutropenia. The mean total leucocyte count for the control group was 15,000 per cubic millimeter, as compared with 10,500 for the pigs receiving the antagonist. The percentage of granulocytes (metamyelocytes, neutrophils, basophils, and eosinophils) was reduced in the antagonist group to 14 as compared with a mean of 47 for the control group. The absolute number of granulocytes in the control animals averaged 7,000 per cubic millimeter of blood, whereas in the animals receiving the antagonist there were only 1,500 per cubic millimeter. Giant metamyelocytes and multinucleated neutrophils such as are found in the blood smears of patients with pernicious anemia were not seen in the blood of the pigs.

The number of platelets per cubic millimeter of blood in the control animals ranged from 310,000 to 510,000. The number of platelets per cubic millimeter of blood in the animals receiving antagonist was extremely variable. Values between 100,000 and 310,000 were frequently obtained, but in one animal (10-64) the number of platelets increased prior to therapy.

Bone Marrow.—In Table IV the results of differential cell counts on sternal marrow obtained from the eight control animals and the four animals receiving antagonist are summarized. In the marrows from the animals receiving antagonist there was a marked reduction in the number of polymorphonuclear neutrophils and neutrophilic metamyelocytes as well as a slight increase in earlier forms of the myeloid series and a significant decrease in the leucocyte-erythroid ratio. In addition, extremely immature and somewhat abnormal nucleated red cells were present (Fig. 7). These cells were round or oval and extremely large, measuring 12 to 25 μ in diameter, whereas the most immature nucleated

TABLE III. DATA ON LEUCOCYTES AND PLATELETS IN LOW PROTEIN CONTROL GROUP AND PGA ANTAGONIST GROUP

		LOW PROTEIN CONTROL					MEAN	PGA ANTAGONIST				MEAN
Pig		10-45	10-46	10-47	10-48	10-50		10-53	10-54	10-56	10-64	
W.B.C. × 1000 (c.mm.)	Mean	15.2	15.6	13.4	16.4	14.6	15.0	8.4	11.9	8.2	13.6	10.5
	Range	14.1-16.2	14.3-17.6	12.7-14.1	15.6-17.3	13.5-15.4	12.7-17.6	7.7-8.8	7.3-18.3	6.4-10.2	9.4-17.1	6.4-18.3
PMN (%)	Mean	44	50	48	48	47	47	8	18	16*	14	14
	Range	41-48	42-53	45-51	40-55	37-50	37-55	1-13	8-23	13-19	10-19	1-23
MNC (%)	Mean	56	50	52	52	56	53	92	82	84*	86	86
	Range	52-59	47-58	49-55	45-60	51-63	45-63	87-99	77-92	81-87	81-90	77-99
PMN × 1000 (c.mm.)	Mean	6.7	7.8	6.4	7.9	6.4	7.0	0.7	2.1	1.3*	1.9	1.5
	Range	5.9-7.5	6.6-9.3	6.7-7.0	6.4-9.1	5.7-7.3	5.7-9.3	0.1-1.1	0.6-3.8	1.1-1.4	0.9-3.2	0.1-3.8
N.R.B.C. per 100 W.B.C.	Mean	1	0	3	0	2	1	11	11	5*	1	7
	Range	0-3	0-1	1-4	0	0,3	0-4	7-15	7-14	5-6	0-1	0-15
Platelets × 1000 (c.mm.)	Mean	460	470	350	410	380	414	307	300*		415	341
	Range	420-510	450-480	430-490	380-460	310-420	310-510	100-680			220-630	100-680

The means represent an average of three separate determinations.

PMN., Polymorphonuclear cells including basophils and eosinophils; MNC, mononuclear cells including both lymphocytes and monocytes; N.R.B.C., nucleated red blood cells.

*Two determinations only.

TABLE IV. SUMMARY OF BONE MARROW STUDIES ON EIGHT LOW PROTEIN CONTROL ANIMALS AND FOUR ANIMALS RECEIVING PGA ANTAGONIST

CELL TYPE	CONTROL		PGA ANTAGONIST	
	MEAN	RANGE	MEAN	RANGE
Myeloblast	0.9	0.4- 1.3	2.0	0.4- 3.4
Promyelocyte	1.6	0.5- 3.8	4.4	2.0- 5.8
N Myelocyte	6.8	2.6-10.0	7.2	4.4-12.4
E Myelocyte	0.8	0.5- 1.4	2.4	1.2- 2.8
N Metamyelocyte	36.6	28.8-46.8	18.0	13.4-25.6
E Metamyelocyte	0.3	0.0- 1.0	1.0	0.0- 1.8
PMN Neutrophil	12.4	5.3-20.6	3.8	2.2- 7.0
PMN Eosinophil	0.2	0.0- 0.6	0.3	0.0- 0.6
Lymphocyte	7.0	4.9- 9.3	3.3	2.0- 5.6
Plasma cell	0.1	0.0- 0.4	0.1	0.0- 0.4
Monocyte	0.5	0.2- 0.9	0.2	0.0- 0.6
Reticulum cell	0.1	0.0- 0.2	1.1	0.0- 2.8
Mitotic cell	0.4	0.0- 0.9	1.4	0.6- 1.8
"Megaloblast"	0.0	0.0	17.3	11.2-22.6
Pronormoblast	0.4	0.0- 1.4	2.2	0.8- 4.2
B Normoblast	1.7	0.2- 3.5	4.7	3.2- 6.4
P Normoblast	28.9	21.0-38.3	29.8	19.6- 45.2
O Normoblast	1.3	0.0- 6.3	0.8	0.2- 1.6
Leucocyte/Erythroid	2.6	1.5- 3.5	0.8	0.6- 1.3

N, Neutrophilic; E, eosinophilic; PMN, polymorphonuclear; B, basophilic; P, polychromatophilic; O, orthochromatic.

red cells seen in the marrows of the control animals measured 6 to 12 microns. These large cells possessed a relatively large nucleus and a somewhat homogeneous basophilic cytoplasm. The nuclei were composed of rather thin meshlike strands of chromatin. In some cells the chromatin showed some tendency to clump; in others the chromatin appeared finely granular and more homogeneous. The more immature of these cells contained two or three distinct nucleoli. A delicate nuclear membrane surrounded the nucleus. Later stages of this cell were present, including the orthochromatic stage.

These cells resembled closely the megaloblastic series of cells seen in the marrow of patients with pernicious anemia, the only distinct difference being that the nuclear chromatin was not so fine and meshlike as that seen in human bone marrow. Whether or not the cells described are pig megaloblasts is a matter for conjecture. In any event, similar cells were not seen in the marrow of the control animals nor have they been observed by us in the marrow of animals following severe and prolonged hemorrhage superimposed on a dietary restriction of iron. For purposes of discussion these cells will be referred to as megaloblasts in this paper in order to distinguish them from cells of the normoblast series. On several occasions orthochromatic megaloblasts were seen in the peripheral blood of the deficient animals.

Plasma Iron, Serum Copper, and Erythrocyte Protoporphyrin.—Plasma iron, serum copper, and erythrocyte protoporphyrin determinations are presented in Table V. These were made at the time of maximal anemia and prior to the initiation of therapy. No significant difference was noted in either the plasma iron or the erythrocyte protoporphyrin values in the two groups; nor were these determinations appreciably different from those obtained in swine maintained on a diet containing 26 per cent casein.[15] Animals maintained on a low protein diet have been found to have a significantly low serum copper.*

*Unpublished data.

The serum copper in two of the animals receiving antagonist (10-53 and 10-64) was low, whereas in the other two (10-54 and 10-56) the values were comparable with those obtained in animals on a 26 per cent casein diet.[15] The significance of this finding is not obvious.

TABLE V. STUDIES ON PLASMA IRON, SERUM COPPER, AND FREE ERYTHROCYTE PROTOPORPHYRIN

GROUP	ANIMAL	P.I. (μg %)	S. Cu (μg %)	E.P. (μg %)
PGA Antagonist	10-53	123	127	79
	10-54	154	220	97
	10-56	118	235	98
	10-64	110	177	100
	Mean	126	190	94
Control	Mean	101	146	108
	Range	66-140	127-179	78-129

P.I., Plasma iron; S.Cu, serum copper; E.P., erythrocyte protoporphyrin in micrograms per 100 ml. red cells.

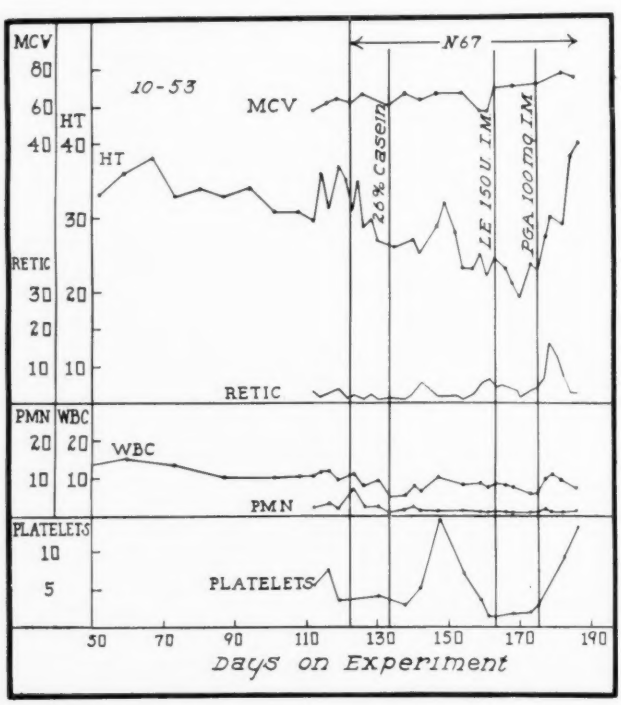

Fig. 1.—Anemia, leucopenia, and neutropenia in a pig (10-53) receiving a pteroylglutamic acid antagonist (N67). There was no hemopoietic response to the administration of casein or purified liver extract (L.E.), but a good response occurred when pteroylglutamic acid (PGA) was given.

M.C.V., Mean corpuscular volume in cubic microns; Ht., volume of packed red cells in ml./100 ml.; Retics., reticulocytes expressed in per cent; W.B.C., total leucocyte count in thousands per c.mm.; PMN, number of polymorphonuclear cells (mature neutrophils, basophils and eosinophils and metamylelocytes) in thousands per c.mm. of blood; Platelets, expressed in hundred thousands per c.mm. of blood; I.M., intramuscular injection. The purified liver extract used contained 15 U.S.P. units per milliliter.

Effect of Therapy on the Peripheral Blood.—One animal (10-53, Fig. 1) was treated twelve days after the administration of antagonist was started by increasing the casein in the diet to 26 per cent. There was a temporary increase

in the volume of packed red cells, but this increase was not sustained and the animal became severely anemic. There was no apparent increase in the total leucocyte or neutrophil counts and there was no significant reticulocytosis. The platelets increased from a low normal value of 300,000 per cubic millimeter to a value considerably above the normal (1,400,000) and then decreased to the thrombocytopenic level of 100,000. The protein therapy had no apparent effect on the mean corpuscular volume. There was, however, a significant growth response (Fig. 5) and a rise in serum albumin from 2.1 Gm. per cent to 3.2 Gm. per cent.

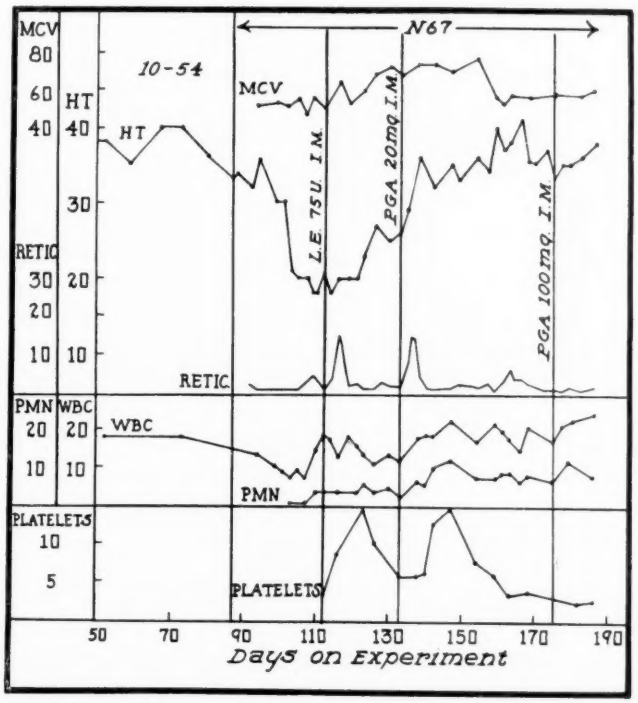

Fig. 2.—Development of anemia, leucopenia, and neutropenia in a pig (10-54) receiving a pteroylglutamic acid antagonist (N67), and response to purified liver extract (L.E.) and pteroylglutamic acid (PGA). For explanation see Fig. 1.

Three animals (10-53, 10-54, 10-56; Figs. 1-3) were treated with purified liver extract prior to pteroylglutamic acid therapy. In one (10-53, Fig. 1) there was no significant effect on any of the blood constituents following the injection of 150 units. In the other two (10-54, 10-56; Figs. 2 and 3) there was a suboptimal response consisting of a reticulocytosis of 15 per cent in one animal (10-54, Fig. 2) and a modest rise in volume of packed red cells and platelets in both. In one pig (10-54, Fig. 2) the total leucocyte count had risen to normal at the time liver therapy was begun. There was no apparent effect from the liver therapy and neutropenia developed again. In the other animal (10-56, Fig. 3) the total leucocyte and neutrophil values rose to normal but were not sustained.

These three animals (10-53, 10-54, 10-56; Figs. 1-3) were then treated with synthetic pteroylglutamic acid and an immediate, rapid, and maximal increase in the volume of packed red cells followed. In two of the pigs (10-53, 10-54; Figs. 1 and 2) a significant reticulocytosis of 16 and 15 per cent, respectively, appeared. An initial rise in the platelet count was observed in two of the animals (10-53, 10-54; Figs. 1 and 2), but in the three (10-54, 10-56, 10-64; Figs. 2-4) in which the platelets were followed for a significant period of time

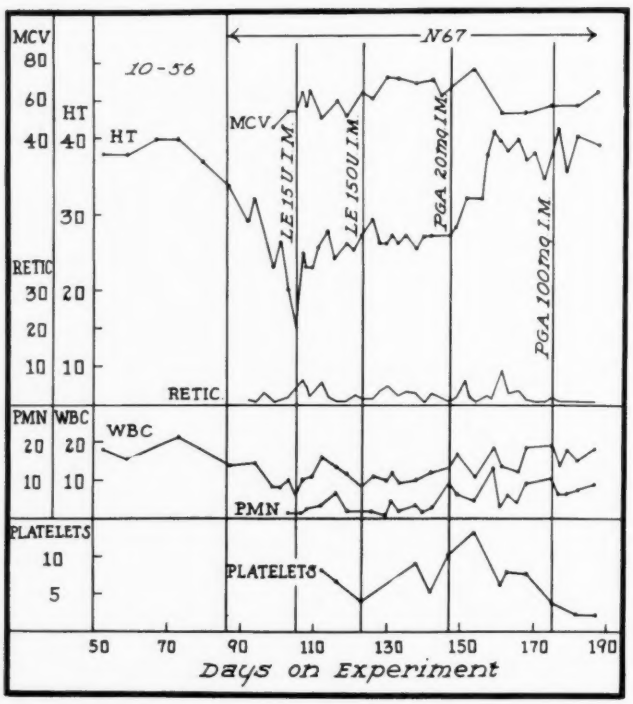

Fig. 3.—Anemia, leucopenia, and neutropenia in a pig (10-56) receiving a pteroylglutamic acid antagonist (N67). There was a questionable response to purified liver extract (L.E.) and a well-marked response to pteroylglutamic acid (PGA). For symbols see Fig. 1.

thrombocytopenia reappeared. In two of the pigs (10-54, 10-56; Figs. 2 and 3) the leucopenia and neutropenia disappeared following pteroylglutamic acid therapy. In the third (10-53, Fig. 1), this effect was not observed and only a slight, transient increase in the total leucocyte count was noted.

All three of the pigs treated initially with liver (Figs. 1-3) developed definite but not marked macrocytosis following liver therapy (Fig. 6). The mean value for the mean corpuscular volume in the control group (twenty-four determinations in five animals) was 56 ± 5.16 cubic microns with a range of 48 to 69 cubic microns. Only twice were the values above 59 cubic microns. In all three of the pigs treated with liver the mean corpuscular volumes reached 72 cubic microns. The macrocytosis could not be attributed to the presence of reticulocytes since these were not significantly increased. In the pigs (Figs. 2 and 3) in which the mean corpuscular volume was followed for a significant

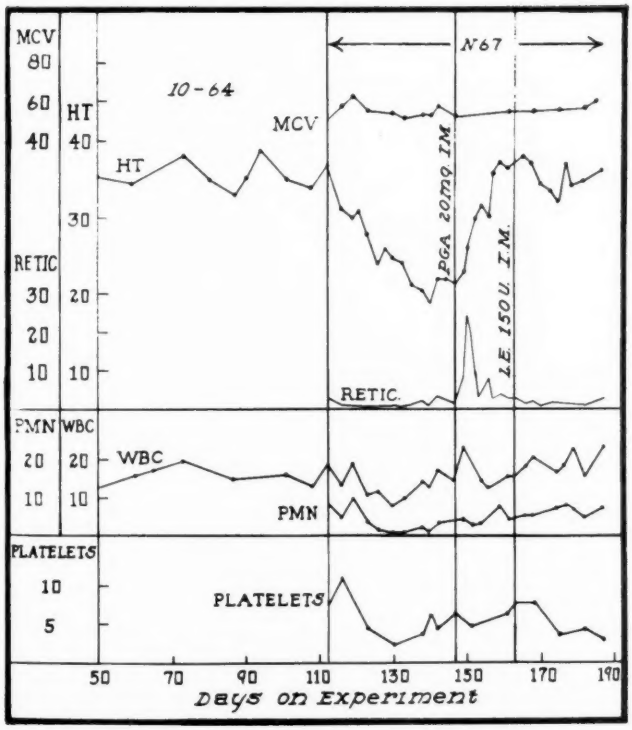

Fig. 4.—Anemia, leucopenia, and neutropenia in a pig (10-64) receiving a pteroylglutamic acid antagonist (N67). There was a pronounced response to pteroylglutamic acid (PGA), but purified liver extract (L.E.) had no effect. For explanation see Fig. 1.

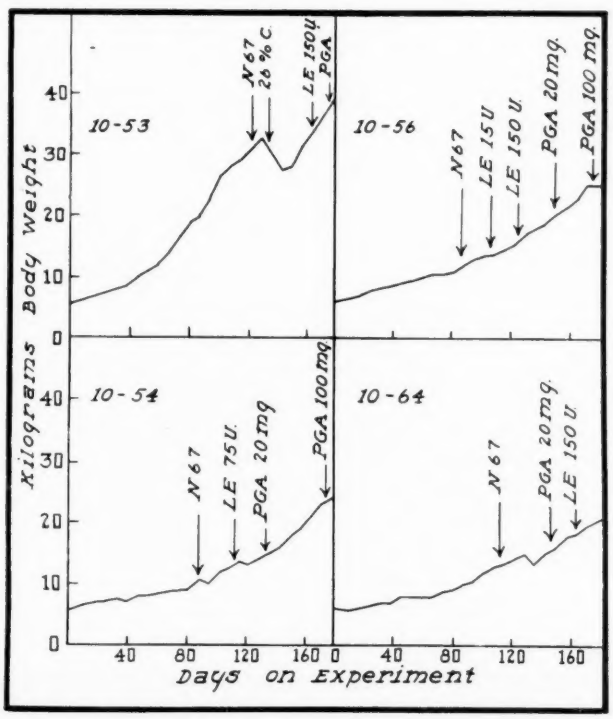

Fig. 5.—Growth curves of the four animals receiving the pteroylglutamic acid antagonist (N67).

period after pteroylglutamic acid therapy the macrocytosis disappeared (Fig. 6). Likewise the anisocytosis and polychromatophilia disappeared, and the Howell-Jolly bodies and nucleated red cells diminished in numbers. This was not the case following liver therapy. As already noted, macrocytosis developed and, in addition, nucleated red cells, Howell-Jolly bodies, and polychromatophilic cells persisted in increased numbers.

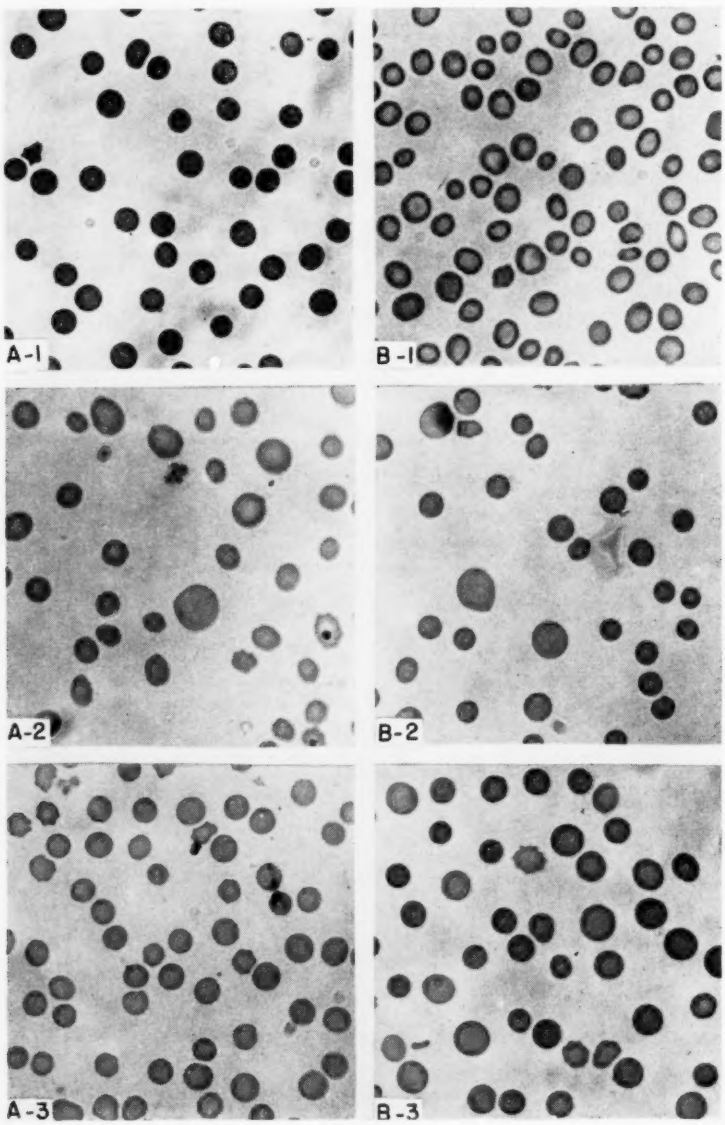

Fig. 6.—Photomicrographs of peripheral blood smears (×1000). A-1, Normal pig (10-50); mean corpuscular volume (M.C.V.) 57 cubic microns. A-2, Pig (10-56) receiving PGA antagonist (139th day of experiment); the smear was made after liver extract had been given and prior to PGA therapy; note marked anisocytosis and numerous macrocytes; M.C.V., 69 cubic microns. A-3, From same animal as in A-2, after PGA therapy (183rd day); note decrease in anisocytosis and absence of macrocytes; M.C.V., 57 cubic microns. B-1, Pig (10-53) prior to administration of antagonist (120th day); M.C.V., 64 cubic microns. B-2, From same pig as in B-1 but after the administration of antagonist (164th day); note marked anisocytosis and numerous macrocytes; M.C.V., 69 cubic microns. B-3, From same pig as in B-2, after liver therapy (187th day); note macrocytosis; M.C.V., 72 cubic microns.

One pig (10-64, Fig. 4) was treated initially with 20 mg. of pteroylglutamic acid. This was followed by an immediate reticulocytosis which reached a maximum of 25 per cent on the third day. At the same time there was a rapid

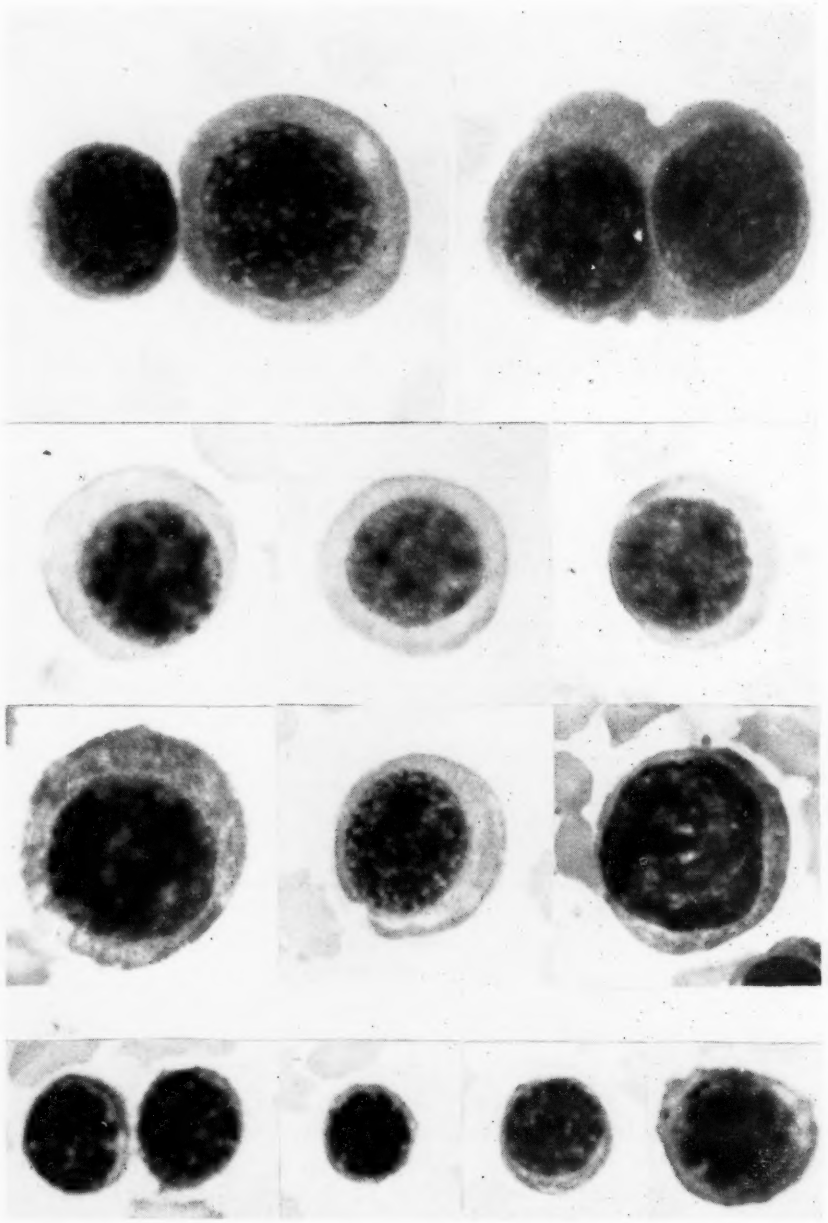

Fig. 7.—Photomicrographs of nucleated red cells (×2900) from the bone marrow of pigs receiving PGA antagonist and of control animals. The cells in the upper three rows are representative of the type of cells seen in the marrows of the animals receiving PGA antagonist and are the cells described in the text as megaloblasts. The cells in the bottom row were taken from the marrows of control pigs. Note the difference in the size of the cells and in the character of the nuclear chromatin.

TABLE VI. BONE MARROW STUDIES ON ANIMALS RECEIVING PGA ANTAGONIST

Pig	10-53				10-54					10-56				10-64				
Days after antagonist	0	37	53	64	22	41	45	74	98	21	39	63	78	0	25	35	50	74
			L.E.	PGA		L.E.		PGA	PGA		L.E.	L.E.	PGA				PGA	L.E.
Therapy			150	100		75		20	100		15	150	20				20	150
Days after therapy			12	11		17	21	29	11		18	24	15				15	23
Myeloblast	0.2	2.2	0.9	1.4	3.4	1.2	0.4	0.2	0.2	0.4	1.4	0.8	1.0	1.8	1.6	2.0	0.4	1.6
Promyelocyte	4.4	5.6	2.3	3.2	5.8	1.8	1.0	3.0	0.8	2.0	3.5	4.2	4.8	2.6	4.5	4.0	2.0	4.0
N Myelocyte	4.2	4.4	1.7	1.8	12.4	5.0	1.4	4.8	2.2	4.4	5.2	9.0	4.8	4.8	10.5	7.6	2.2	7.2
E Myelocyte	1.2	2.8	0.7	0.4	2.8	2.2	2.6	1.0	0.0	1.2	1.1	0.0	1.4	2.0	0.3	2.8	0.2	0.8
N Metamyelocyte	30.2	13.4	11.6	12.6	18.6	36.8	15.2	21.8	29.6	14.4	33.0	36.3	39.0	29.8	29.1	25.6	19.8	29.6
E Metamyelocyte	1.6	1.6	0.9	0.0	0.0	1.0	1.2	1.2	1.2	0.4	0.6	0.0	0.8	0.2	0.6	1.8	1.0	0.8
PMN Neutrophil	8.0	3.6	4.7	6.0	2.2	8.2	11.8	5.4	16.0	2.8	9.9	9.5	9.6	14.2	3.5	7.0	13.4	8.4
PMN Eosinophil	0.2	0.6	0.2	0.0	0.0	0.4	0.8	0.0	0.6	0.4	1.1	4.7	0.4	0.2	0.0	0.2	0.2	0.6
Lymphocyte	4.8	2.6	6.9	40.8	3.0	6.4	10.0	2.8	10.0	5.6	3.5	9.0	9.8	7.2	12.7	2.0	17.4	15.6
Plasma cell	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Monocyte	0.2	0.0	1.6	4.0	0.0	0.8	0.6	0.2	2.0	0.4	0.5	0.0	0.8	0.6	1.5	0.6	0.6	1.6
Reticulum cell	0.0	0.0	0.9	0.0	1.6	0.0	0.0	0.0	0.0	0.0	0.5	0.3	0.0	0.2	0.1	2.8	0.2	0.0
Mitotic cells	0.0	1.8	1.6	0.2	1.4	0.8	1.2	0.2	0.0	1.6	1.0	0.2	0.2	0.0	0.5	0.6	0.4	0.0
"Megaloblast"	0.0	22.6	12.4	0.0	20.2	4.2	3.2	0.4	0.0	11.2	5.7	1.5	0.0	0.0	9.0	15.2	1.4	0.4
Pronormoblast	0.4	2.2	2.7	0.2	4.2	5.2	0.8	0.4	0.0	1.6	0.6	1.3	0.2	1.0	0.8	0.8	0.4	0.4
B Normoblast	4.4	5.2	6.8	2.8	3.8	6.4	2.4	12.6	2.0	6.4	5.1	7.5	2.4	1.0	4.3	3.2	2.8	3.8
P Normoblast	38.0	30.8	42.3	25.6	19.6	17.2	44.8	44.2	35.4	45.2	27.0	13.0	25.0	32.6	20.9	23.4	35.0	24.2
O Normoblast	1.8	0.6	1.6	1.0	1.0	2.8	2.6	1.6	0.0	1.6	0.3	2.7	0.0	1.8	0.1	0.2	2.6	1.0
Leucocyte/Erythroid ratio	1.2	0.6	0.5	2.4	1.0	1.8	0.9	0.7	1.7	0.5	1.6	2.8	2.6	1.7	1.8	1.3	1.4	2.4

N, Neutrophilic; E, eosinophilic; PMN, polymorphonuclear; B, basophilic; P, polychromatophilic; O, orthochromatic; L.E., Liver extract (15 units per cubic centimeter) in units; PGA, pteroylglutamic acid in milligrams.

rise in the volume of packed red cells to the level observed in pigs on the low protein diet, and an increase in the leucocyte and neutrophil count occurred. There was no apparent increase in platelets. A secondary reticulocytosis was not elicited by the administration of 150 units of liver extract and no further increase in the volume of packed red cells was noted. A significant macrocytosis was not present in this animal at any time.

The effects of the various therapeutic agents on growth are shown in Fig. 5.

Effect of Therapy on the Bone Marrow.—The effects of therapy on the bone marrow of each of the animals receiving the antagonist are presented in detail in Table VI.

The administration of liver extract was associated with a diminution in the number of megaloblasts in the marrow in each instance (Pigs 10-53, 10-54, and 10-56), but did not result in a complete disappearance of these cells. Cells were seen following liver therapy which had characteristics intermediate between basophilic normoblasts and megaloblasts. In two of the animals (10-53 and 10-54) the leucocyte-erythroid ratio became more abnormal following liver therapy, whereas in the third (10-56) the ratio reverted to normal. In one pig (10-53) liver therapy had no apparent effect on the maturation of the leucocytes; in another (10-54) there was a significant increase in metamyelocytes followed later by an increase in neutrophils, whereas in the third there was a marked increase in metamyelocytes and a significant increase in neutrophils. In general, the changes in the bone marrow following liver therapy were consistent with those seen in the peripheral blood.

Pteroylglutamic acid therapy resulted in a restoration of the bone marrow to normal in three instances. In one pig (10-53), although the megaloblasts disappeared completely and the leucocyte-erythroid ratio returned to normal, there was an increase in the percentage of lymphocytes and a decrease in metamyelocytes and neutrophils. In this animal the leucopenia and neutropenia in the peripheral blood persisted in spite of therapy.

At the termination of the experiment the animals were sacrificed and autopsies performed. There was no splenic enlargement and the marrow in the sternum, ribs, vertebrae, and femurs was red and hyperplastic. There were no significant microscopic abnormalities in any of the organs examined which could not be attributed to a deficiency of protein.

DISCUSSION

The observations presented here indicate that there is a marked difference between the effect of pteroylglutamic acid and that of liver extract of the pernicious anemia type in pigs fed low protein diets and receiving a folic acid antagonist. The administration of liver extract was associated with a good reticulocyte response and a modest rise in volume of packed red cells in one animal (10-54), but in another (10-56) the effect was less impressive, and in a third (10-53) no significant change was observed in the blood even though a very large amount of liver extract, 150 units, was given. The administration of pteroylglutamic acid, on the other hand, was associated with a well-pronounced hemopoietic response in these three animals as well as in another (10-64) not previously treated with liver extract.

These results are not in accord with the observations of Heinle, Welch, and co-workers[7] who concluded that "extrinsic factor and purified liver extracts are effective even though folic acid is not available to the animal." There are, however, several differences in the experimental conditions employed by the Cleveland group and by ourselves. Our animals received a diet low in protein. Presumably the three pigs reported by Welch, Heinle, and colleagues received adequate amounts of protein. Furthermore, their animals were given Labco* "vitamin-free" casein which contains little or no extrinsic factor,[18] while our animals were fed casein proved to contain substantial quantities of extrinsic factor. Finally, our animals were depleted of pteroylglutamic acid for a prolonged period prior to the administration of antagonist. Whether these differences in experimental procedure are significant or important cannot be stated at this time. It should be noted that the response of the one animal reported in detail by the Cleveland group was, for a pig, considerably delayed and gradual.[6]

From one point of view it may be considered surprising that any hemopoietic response to liver extract should have been observed in our animals. There is no reason to assume that there was in these animals a deficiency of intrinsic factor, although this was not specifically investigated. They were fed what would seem to be, at least in terms of treating a patient with pernicious anemia, a rather substantial amount of extrinsic factor. One might ask whether under the conditions of the experiment, when a low protein diet, sulfasuxidine, and antagonist were administered, our animals actually absorbed an adequate amount of extrinsic factor. Since diarrhea was present, absorption may not have been complete. The theoretic though remote possibility that extrinsic factor can be synthesized in the intestinal tract by bacteria must not be overlooked. If this were true, the administration of both sulfasuxidine and antagonist might seriously hinder such synthesis. In favor of the existence of a double deficiency, that is both of extrinsic factor and of pteroylglutamic acid, is the fact that in the animal treated first with protein (10-53) there was no significant response to the subsequent administration of purified liver extract. However, contradicting this hypothesis is the fact that Pig 10-64 responded maximally to 20 mg. of pteroylglutamic acid without prior feeding of increased amounts of casein. Furthermore, the administration of 150 units of liver extract sixteen days later failed to produce a secondary response. It may be mentioned here that in other experiments with pigs maintained on a low protein diet which were repeatedly depleted of niacin, we have observed no response to liver extract.[19]

A more plausible explanation of the activity of liver extract, such as it was, would be that a substance which it contains is concerned in some way with the utilization or availability of pteroylglutamic acid and that the administration of the large doses of liver extract made small tissue stores of pteroylheptaglutamic acid available to the animal or that pteroylglutamic acid was released from more complex compounds. In patients with pernicious anemia in relapse, Bethell and co-workers[16] as well as Welch and associates[1, 17] have observed that a substance in purified liver extract may contribute to the utilization of con-

*The Borden Co., New York, N. Y.

jugates of folic acid. The possibility that liver extract makes small amounts of pteroylglutamic acid available from the tissues is now under investigation in the current animal experiment.

Since the doses of liver extract used were large (75 to 150 units), it is unlikely that their unimpressive effect was due to inadequate dosage. The response which seemed to occur probably cannot be explained on the basis of the pteroylglutamic acid content of the liver extract since it has been shown that this preparation contains less than 1.0 μg of microbiologically determinable *L. casei* factor per milliliter.[8] Furthermore, if the response was due to pteroylglutamic acid or its precursors in the liver extract, Pig 10-56 should have responded when 10 ml. of liver extract were administered. Instead, there was a slight initial response to 1 ml. of liver extract and no further response when 10 ml. were given eighteen days later. Again, the complete lack of response to 150 units in Pig 10-53 is noteworthy.

It is of interest that the anemia observed was normocytic at first. Smears of the peripheral blood during this period revealed numerous large macrocytes and a comparable number of microcytes. After liver therapy in three animals definite macrocytosis appeared and this persisted beyond the period of reticulocytosis. It seems unlikely that the macrocytosis was due to liver therapy although this possibility has not been ruled out. A more plausible explanation would seem to be that sufficient time had not elapsed for a significant degree of macrocytosis to develop which would be manifest when mean values were determined. Provided the life span of the red cell of the pig is similar to that of man, that is about 120 days, replacement of the circulating, normal-sized red corpuscles by macrocytes, with a corresponding increase in the mean corpuscular volume, could not be expected in twenty to forty days. Since the liver had little or no effect on the anemia, a further period of forty-six to sixty-one days was available after liver therapy during which macrocytosis might develop. Although it is true that the one animal not treated initially with liver failed to develop macrocytosis, it must be noted that this animal received the antagonist for only thirty-five days prior to the administration of pteroylglutamic acid. It is of interest that the one pig reported in detail by Welch, Heinle, and co-workers[6] did not develop significant macrocytosis until the post-treatment period.

The response of the leucocytes to liver extract and pteroylglutamic acid therapy is difficult to interpret. In several instances the values appeared to be rising at the time therapy was begun. In one animal there was no response to either substance. Although the anemia of the pig reported by Welch and associates[6] responded to the therapy given, the leucocyte and neutrophil counts did not return to normal. It seems possible that a substance other than pteroylglutamic acid or the antipernicious anemia factor is involved. The platelet values in our animals are also exceedingly difficult to interpret. There appeared to be a transient thrombocytosis in association with protein, liver, or pteroylglutamic acid therapy, followed by a decrease to an abnormally low level. More information is needed before definite conclusions can be drawn concerning the leucocytes and platelets.

There are various possible explanations of the relationship of liver extract factor and pteroylglutamic acid in hemopoiesis. It has been pointed out already, for example, that the liver factor may be concerned with the liberation of pteroylglutamic acid from conjugated forms of this substance in the tissue stores. If this were true, one would expect both liver extract and pteroylglutamic acid, the substance it makes available, to be effective in producing a hemopoietic response in pernicious anemia. One would also expect that pteroylglutamic acid deficiency, if this included a deficiency of conjugates as well as of free forms of the vitamin, would not be affected by the administration of liver extract. If a deficiency of extrinsic factor were produced, one would expect a response to occur when extrinsic factor or liver extract or pteroylglutamic acid was given.

Such an hypothesis would be consistent with our own report[20] in which an anemic pig fed a highly purified diet responded to the administration of liver extract, if we assume that that animal was deficient in extrinsic factor and was, at most, only partially deficient in pteroylglutamic acid and its conjugates; it would explain the findings of Welch, Heinle, and co-workers[6, 7] if it can be assumed that their animals were deficient in extrinsic factor and only partially deficient in pteroylglutamic acid and its conjugates; and it would be satisfied by our own observations, presented in this report, if it is assumed that our animals were mainly deficient in pteroylglutamic acid and its conjugates. The unimpressive effect of liver extracts and the variations in the different animals could be explained by assuming that only a moderate or slight deficiency of extrinsic factor was produced in our animals when a crude casein containing extrinsic factor was fed in low amounts.

Totter, Sims, and Day,[21] on the basis of indirect evidence, have suggested that pteroylglutamic acid is concerned with the synthesis of protoporphyrin. The data on free erythrocyte protoporphyrin presented here do not substantiate this but admittedly do not disprove the theory.

It may be mentioned, in closing, that a pronounced ataxia appeared in one animal (10-64) approximately ten days after the administration of 20 mg. of pteroylglutamic acid. A similar phenomenon has been observed in animals fed a diet deficient only in protein. This will be the subject of a separate report.

SUMMARY

Four pigs were maintained on a synthetic diet containing 10 per cent crude casein which was shown to possess extrinsic factor activity. The diet was supplemented with various vitamins, exclusive of pteroylglutamic acid, p-aminobenzoic acid, and inositol; sulfasuxidine and a pteroylglutamic acid antagonist were given as well. A severe anemia, leucopenia, and neutropenia developed. No such changes were observed in the blood of eight control animals maintained on the same diet plus pteroylglutamic acid, p-aminobenzoic acid, and inositol and to which no sulfasuxidine or pteroylglutamic acid antagonist was added.

Studies on the bone marrow of the animals receiving the pteroylglutamic acid antagonist revealed a marked reduction in the number of polymorphonuclear neutrophils and neutrophilic metamyelocytes, with a slight increase in the earlier forms of the myeloid series and a significant decrease in the leucocyte-erythroid

ratio. In addition, immature and nucleated red cells in many ways similar to those seen in the bone marrow of patients with pernicious anemia in relapse were observed.

The anemia became more pronounced in spite of a great increase in the protein content of the diet of one animal. It responded partially to the administration of liver extract in one animal, questionably in another, and not at all in a third. Rapid relief of the anemia followed the administration of small doses of pteroylglutamic acid in all four pigs.

Macrocytosis developed as the anemia progressed but at first the mean corpuscular volume was normal, probably because of the presence of many microcytes. In the later stages of the disorder macrocytic anemia was demonstrated, many more macrocytes than microcytes being present.

The leucopenia and neutropenia were not permanently relieved by either protein or liver therapy. In three of the animals the leucopenia and neutropenia were no longer present following the administration of pteroylglutamic acid. In one animal the leucopenia and neutropenia persisted even after protein, liver, and pteroylglutamic acid were given. The platelets increased markedly after the administration of protein, liver, or pteroylglutamic acid, but the increased levels were not sustained.

The data suggest that the antipernicious anemia substance in purified liver extract does not completely, if at all, replace pteroylglutamic acid in the nutrition of the pig.

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A STUDY OF THE RESPONSE OF BACTERIAL POPULATIONS TO THE ACTION OF PENICILLIN; A QUANTITATIVE DETERMINATION OF ITS EFFECT ON THE ORGANISMS

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THE action of penicillin on susceptible bacteria is still unknown despite the intense effort that is being made to discover the mechanism of its antibacterial action. While we fully realize the importance of a knowledge of the modus operandi of this valuable therapeutic agent, we believe it equally important, especially from the clinical standpoint, to know what actually happens to the bacteria when exposed to the action of the drug. The first observation by Fleming[1] which led to the discovery of penicillin was that of lysis of staphylococci or destruction of organisms, and yet the action of the antibiotic has been referred to since as bacteriostatic rather than bactericidal. The sensitivity tests on bacteria, as performed today, are evidently directed toward the determination of the inhibitory activity of the penicillin on the microorganisms. Even in assaying the antibiotics, it is the inhibitory activity of the material on the standard test organism which is being considered. Results of penicillin sensitivity tests are reported in such terms as the minimum inhibiting concentration, the titer of inhibition, the minimum effective concentration, and so forth. Some workers using a standard organism in their sensitivity tests compare the titer of the test with that of the standard and report their results as half as sensitive as the standard, one-quarter as sensitive, and so on. Such reports only vaguely suggest the action of the drug on the bacteria. Although several accounts of the bactericidal effect of penicillin have recently been reported,[2-7] the action is still referred to as bacteriostatic, or interchangeably referred to as bacteriostatic and bactericidal.

It is the purpose of this communication to present experiments which show that it is possible to determine the exact condition of a bacterial population when exposed to the action of penicillin—the degree of sterilization, the number killed and the number surviving, the character of the survivors—and to discuss how these findings may influence and even lead to a modification of present-day treatment of infectious diseases with the antibiotics.

MATERIAL AND METHODS

The organisms used in the experiments were pneumococci from cases of lobar pneumonia, hemolytic streptococci from scarlet fever and erysipelas, *Streptococcus viridans* strains from cases of subacute bacterial endocarditis, and staphylococci from general septicemias. All except one strain (*Str. viridans* e) were initial specimens, that is they were obtained from patients before penicillin treatment was instituted. The organisms were grown in phosphate-buffered beef heart broth, enriched when necessary with 3 per cent citrated horse plasma. Bacterial counts were made in nutrient agar poured plates, 3 per cent of citrated horse blood being added to the agar.

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Commercial sodium penicillin* and streptomycin hydrochloride† were employed in the sensitivity tests, and penicillinase‡ was used to inactivate the penicillin in the cultures.

Of the several methods now in use for determining the sensitivity of bacteria to the antibiotic, the serial dilution method is considered to be the most reliable since it allows a wide range of penicillin concentrations. This method, briefly, is as follows. Twofold serial dilutions of penicillin, using broth as diluent, are made in small tubes. The number of tubes set up in a test depends on the range of penicillin concentrations desired, usually twelve. The penicillin dilutions are contained in 0.5 ml. of broth. To each tube 0.5 ml. of a young culture, a five- to six-hour growth, diluted to 10^{-5} is added, giving a total of 1 ml. in each tube. One tube containing culture without penicillin is added as control. The tubes are incubated at 37° C. and are read the following day. The last clear tube in the series containing the least amount of penicillin which "inhibits" bacterial growth as judged by visual inspection is regarded as the indicator of the penicillin sensitivity of the organism tested. Two streaked plates are made from the last two clear tubes to "insure sterility."

Since visible lysis is a direct proof of destruction of bacteria, we decided first to subject each culture to such a test. For this purpose an opaque culture is needed to observe clearing. Diluting the culture, as is done in the described procedure, would not do, since the inoculated tubes look quite clear before incubation. Too many organisms (overnight growth), on the other hand, would obscure lysis if it occurred only to a slight degree. A three- to four-hour culture, depending on the rate of growth of the particular organism, gave the desired turbidity and was used undiluted. The same culture was also tested when diluted to 10^{-4} to compare the sensitivity titers. The tenfold serial dilutions were used in agar poured plates for bacterial counts. The number of organisms in the inoculum in different tests generally varied from 100,000,000 to 300,000,000 in the straight culture and from 10,000 to 30,000 in the diluted culture although smaller numbers were occasionally employed. The turbidity of the cultures was read before incubation, and one tube was placed in the refrigerator to compare directly with the incubated tubes the following morning, as a check on the turbidity reading. The tubes were incubated at 37° C. for eighteen hours. The turbidity of the cultures was read by comparing each tube with a standard consisting of a set of fuller's earth suspensions in tubes ranging from a slight turbidity (25) to a density through which a dark object barely could be seen (500), twenty tubes in all. The titer of sensitivity, when straight culture was used for inoculation, was considered the smallest amount of penicillin which gave a turbidity reading equal to that of the initial culture before incubation.

Knowing that minute amounts of penicillin may inhibit growth of sensitive bacteria, we suspected that the inoculum used to subculture in normal media for sterility tests carried with it sufficient penicillin to inhibit growth and thus cause faulty interpretation of results. In order to eliminate this possibility we diluted the cultures exposed to penicillin so that the final concentration of the drug in the poured plates was reduced to negligible amounts. Penicillinase, used to inactivate the penicillin in the cultures, gave results similar to those obtained by dilution, and, since it was more convenient, this method was employed routinely for bacterial counts in 1 unit per milliliter amounts.

RESULTS OF THE EXPERIMENTS

As can be seen from Table I, the destruction of organisms by lysis occurred in all the bacterial strains tested except four, those of *Str. viridans* strains identified as *salivarius*. Two greening streptococcus strains, one an enterococcus and the other unidentified, were lysed but in higher concentrations of penicillin. The extent of bacteriolysis varied in the different species, pneumococci showing the most, staphylococci less, and hemolytic streptococci least.

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†Merck and Company, Inc., Rahway, N. J.
‡Schenley Laboratories, Inc., New York, N. Y.

TABLE I. PENICILLIN SENSITIVITY TESTS SHOWING LYSIS OF ORGANISMS; TURBIDITY READING AFTER 18-HOUR INCUBATION AT 37° C.

ORGANISM	TURBIDITY BEFORE INCUBATION	PENICILLIN DILUTIONS BEGINNING WITH 60 UNITS/ML.													INCUBATED CONTROL (NO PENICILLIN)
		60	30	15	7.5	3.75	1.87	0.93	0.46	0.23	0.12	0.06	0.03	0.015	
Str. viridans a	50	50	50	50	50	50	50	50	50*	75	75	75	150	175	225
Str. viridans b	50	50	50	50	50	50	50	50	50*	75	75	100	125	150	200
Str. viridans c	50	50	50	50	50	50	50	50	50*	75	75	75	100	150	200
Str. viridans d	50	50	50	50	50	50	50	50	50*	75	100	125	150	175	175
Str. viridans e	75	tr.	tr.	tr.	tr.	tr.	50*	425	425	450	500	500	500	500	500
Enterococcus	75	50	50	50	50*	150	200	225	250	275	275	275	275	275	275
Staph. aureus	75	25	25	25	25	25	25	25	25	25	25	25	75*	275	500
Staph. citreus	50	25	25	25	25	25	25	25	25	25	25	50*	75	225	500
Hem. strept. 98	75	50	50	50	50	50	50	50	50	50	75*	100	125	150	200
Hem. strept. 11	100	75	75	75	75	75	75	75	75	75	75	100*	125	150	225

ORGANISM	TURBIDITY BEFORE INCUBATION	PENICILLIN DILUTIONS BEGINNING WITH 20 UNITS/ML.												INCUBATED CONTROL (NO PENICILLIN)
		20	10	5	2.5	1.25	0.63	0.32	0.16	0.08	0.04	0.02	0.01	0.005	
Pneumo. Type 1	100	25	25	25	25	25	25	25	25	25	25	25	50*	200	225
Pneumo. Type 2	75	25	25	25	25	25	25	25	25	25	25	50*	100	200	225
Pneumo. Type 3	125	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	25	50*	200	225
Pneumo. Type 1a	100	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	25	50	100*	225
Pneumo. Type 8	100	25	25	25	25	25	25	25	25	25	25	50*	125	250	450
Pneumo. Type 9	50	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	25	50*	225

tr., Trace.
*Titer of macroscopic inhibition.

Individual strains within the same species also differed but within a smaller margin. Lysis was not complete in any of the cultures, a residuum of viable cells always remaining even in the highest concentration of penicillin used, that of 60 units per milliliter. In most instances the number of organisms surviving varied inversely with the degree of lysis.

Avery and Cullen[8] have shown that pneumococcal autolysin, while having no action on living pneumococci, has a powerful lytic action on heat-killed pneumococci. Bronfenbrenner and Muckenfuss[9] have shown the same to be true of staphylococci. It is, therefore, difficult to tell whether the lysis of these organisms was caused by the penicillin or whether the bacteria were first killed by the antibiotic and then autolyzed. It is possible that the lysis of pneumococci and staphylococci was a summation of effects of unfavorable influences on the bacterial cell. The lysis of hemolytic streptococci suggests that penicillin is capable of lysing bacteria by itself, since these organisms do not autolyze. Attempts to prepare autolysin from hemolytic streptococci have been unsuccessful.[10]

Table II represents a typical experiment performed with a *Str. viridans* strain. When the penicillin cultures were subcultured in normal media no growth appeared in the plates. Enough of the penicillin was evidently left in the tubes to still inhibit growth of the bacteria. However, when the penicillin in the tubes was inactivated with penicillinase and poured plates made, vast numbers of organisms were recovered, even from the tube containing 60 units per milliliter or 7,500 times as much as the titer of "inhibition" (0.008 unit per milliliter).

When undiluted straight cultures used in the initial inoculum yielded a large number of residual organisms, a proportional number of viable bacteria were recovered from the penicillin cultures in which diluted inoculum was employed. In more sensitive strains, on the other hand, in those yielding smaller numbers of residual organisms, no viable cells were found on subculture when a diluted inoculum was used. These findings indicate that individual organisms in a bacterial strain may differ in their reaction to the effect of the antibiotic. When cells of a certain type are present in small numbers they will be eliminated by high dilutions. Therefore, the cells capable of withstanding large amounts of penicillin, being present in insufficient numbers to be carried through the serial dilutions, were excluded when the culture was diluted ten thousand times (10^{-4}). See Tables II and III.

Higher titers of inhibition were obtained with diluted rather than with undiluted cultures. This did not, however, indicate the true extent of susceptibility of the bacterial strain tested. A comparison of Tables II and III demonstrates this clearly. Here the sensitivity of a hemolytic streptococcus may be compared with that of a *Str. viridans* strain. Both are susceptible to the same extent, according to the dilution method. However, when the penicillin in the tubes was inactivated with penicillinase and bacterial counts were made, much greater numbers of viable organisms were recovered in the *Str. viridans* strain than in the hemolytic streptococcus strain—23 per cent

TABLE II. REPRESENTATIVE TEST WITH A STR. VIRIDANS STRAIN (A) SHOWING NO GROWTH IN PENICILLIN CULTURES WITHOUT PENICILLINASE AND GROWTH WHEN THE DRUG WAS INACTIVATED WITH PENICILLINASE

CULTURES EXPOSED TO PENICILLIN (UNITS/ML.)	CULTURAL PROCEDURE: NUMBER OF ORGANISMS INOCULATED: 30,000,000			3,000			TURBIDITY: NUMBER OF ORGANISMS INOCULATED	
	STREAKED PLATES	POURED PLATES WITHOUT PENICILLINASE	POURED PLATES WITH PENICILLINASE	STREAKED PLATES	POURED PLATES WITHOUT PENICILLINASE	POURED PLATES WITH PENICILLINASE	30,000,000 CULTURE UNDILUTED	3,000 CULTURE DILUTED TO 10^{-4}
60	–	–	7,000,000 colonies*	–	–	724 colonies	50	0
30	–	–	Not counted	–	–	816 colonies	50	0
15	–	–	Not counted	–	–	778 colonies	50	0
7.5	–	–	Not counted	–	–	952 colonies	50	0
3.75	–	–	Not counted	–	–	956 colonies	50	0
1.87	–	–	Not counted	–	–	938 colonies	50	0
0.93	–	–	Not counted	–	–	880 colonies	50	0
0.46	–	–	13,000,000 colonies*	–	–	920 colonies	50	0
0.23	–	–	Not counted	–	–	Not counted	75	0
0.12	–	–	Not counted	–	–	Not counted	75	0
0.06	+	+	Not counted	–	–	Not counted	75	0
0.03	+	+	Not counted	–	–	Not counted	150	0
0.015	+	+	Not counted	–	–	Not counted	175	0
0.008	+	+	Not counted	–	–	880 colonies	200	0
0.004	+	+	Not counted	+	+	Not counted	225	175
No penicillin, before incubation							50	0
No penicillin, after incubation							225	175

Bacterial counts subject to inherent error of method.
–, No growth; +, growth.
*Serial diluted for counting.

TABLE III. REPRESENTATIVE TEST WITH A HEMOLYTIC STREPTOCOCCUS STRAIN (98) IN WHICH NO VIABLE ORGANISMS WERE FOUND WHEN INOCULUM WAS DILUTED TO 10^{-4} BECAUSE OF SMALL YIELD OF RESIDUAL CELLS

CULTURES EXPOSED TO PENICILLIN (UNITS/ML.)	CULTURAL PROCEDURE						TURBIDITY	
	NUMBER OF ORGANISMS INOCULATED						NUMBER OF ORGANISMS INOCULATED	
	300,000,000			30,000			300,000,000	30,000
	STREAKED PLATES	POURED PLATES WITHOUT PENICILLINASE	POURED PLATES WITH PENICILLINASE	STREAKED PLATES	POURED PLATES WITHOUT PENICILLINASE	POURED PLATES WITH PENICILLINASE	CULTURE UNDILUTED	CULTURE DILUTED TO 10^{-4}
60	–	–	7,000 colonies*	–	–	–	50	0
30	–	–	Not counted	–	–	–	50	0
15	–	–	Not counted	–	–	–	50	0
7.5	–	–	Not counted	–	–	–	50	0
3.75	–	–	Not counted	–	–	–	50	0
1.87	–	–	Not counted	–	–	–	50	0
0.93	–	–	Not counted	–	–	–	50	0
0.46	–	–	Not counted	–	–	–	50	0
0.23	–	–	140,000 colonies*	–	–	–	50	0
0.12		5 colonies	Not counted	–	–	–	75	0
0.06			Not counted	–	–	–	100	0
0.03			Not counted	–	–	–	125	0
0.015			Not counted	–	–	–	150	0
0.008			Not counted	–	–	15 colonies	175	0
0.004			Not counted				200	200
No penicillin, before incubation							75	0
No penicillin, after incubation							200	200

–, No growth.
*Serial diluted for counting.

survivals in the former as compared with a small fraction of 1 per cent in the latter. These facts may account for the general observation that bacteria, tested by the present-day methods, show a greater sensitivity to penicillin in vitro than in vivo. The use of straight undiluted cultures in the tests gives results more approximating the sensitivity of bacteria in vivo.

The residual viable bacteria were found to be inhibited in low as well as in high concentrations of penicillin, showing a wide range of inhibition. This was more evident in the tests in which the cultures used for inoculation were diluted. (See Table II.) It is probably due to the elimination of certain cells by the dilution, leaving a more uniform population.

Residual organisms (single colony culture) when retested, that is on second exposure to penicillin, showed no increase in resistance, or only a slight increase, and again yielded a residuum of viable cells similar to that of the original or parent strain. Lysis of cells, however, either did not occur, or occurred to a lesser degree than in the original culture. This again demonstrates the singular characteristic these residual cells have of being able to withstand the killing effect of the drug in high as well as in low concentration.

Since these findings point to the existence in a bacterial population of members differing in the manner in which they respond to the action of penicillin, it was thought advisable to test the residual bacteria which are capable of withstanding high concentrations of the drug with streptomycin, in the hope that they might be susceptible to the latter antibiotic. For this purpose the following method was adopted.

Nutrient agar plates are seeded with an overnight growth of the culture to be tested by placing 0.3 ml. of the broth culture in the center of the plate and spreading it with a wire spreader so that an even, uniform film is produced. The plates are placed in the incubator for fifteen minutes with covers tilted for drying. Paper disks* are impregnated with high concentrations of the drugs. A sterile pointed forceps is used in handling the disks. Each disk is immersed in the solution, the excess being drained off against the wall of the tube. The disks are carefully placed on the surface of the inoculated plate, four to a plate: two disks with penicillin solution 100 units per milliliter, one disk with streptomycin 100 units per milliliter, and one disk with plain broth for control.

The following morning the zones of inhibition are measured and the paper disks used for residual organisms as follows. With sterile forceps each disk is carefully picked up and each placed into 10 ml. of sterile broth. The tubes are well shaken to free the bacteria which adhere to the paper disks. The original concentration of penicillin is thus diluted tenfold and by plating 1 ml. of the washing it is further diluted so that the final concentration in the plate is about one-hundredth that of the original concentration in the disk, not considering the amount of the drug which was absorbed by the agar. If the disk does contain some remaining penicillin it is in negligible amount so that the possibility of its acting on the subculture may be excluded.

*Whatman filter paper No. 2; disk 10 mm. in diameter.

TABLE IV. EFFECT OF STREPTOMYCIN ON PENICILLIN-RESIDUAL BACTERIA
(PAPER DISKS* IMPREGNATED WITH SOLUTION OF 100 UNITS/ML. OF THE DRUGS)

BACTERIAL STRAIN	RESIDUUM CARRIED BY DISK WITH DRUG	NUMBER OF ORGANISMS RECOVERED	ZONE OF INHIBITION	ORGANISMS CARRIED BY DISK WITHOUT DRUG (NORMAL CULTURE)	NUMBER OF ORGANISMS OBTAINED BY SERIAL DILUTION		
					10^{-3}	10^{-4}	10^{-5}
Str. viridans a	Penicillin disk	14,000	32 mm.	Normal culture	650	80	11
	Streptomycin disk	Innumerable	None	Normal culture exposed to streptomycin, 10 units/ml.	652	69	8
	Penicillin residuum exposed to streptomycin, 10 units/ml.	0					
Str. viridans b	Penicillin disk	3,166	29 mm.	Normal culture	586	72	8
	Streptomycin disk	Innumerable	None	Normal culture exposed to streptomycin, 10 units/ml.	592	62	2
	Penicillin residuum exposed to streptomycin, 10 units/ml.	0					
Str. viridans c	Penicillin disk	1,363	28 mm.	Normal culture	346	33	4
	Streptomycin disk	Innumerable	None	Normal culture exposed to streptomycin, 10 units/ml.	300	23	5
	Penicillin residuum exposed to streptomycin, 10 units/ml.	4					
Str. viridans e	Penicillin disk	60,000	14 mm.	Normal culture	188	18	0
	Streptomycin disk	Innumerable	None	Normal culture exposed to streptomycin, 10 units/ml.	188	15	3
	Penicillin residuum exposed to streptomycin, 10 units/ml.	60,000					
Enterococcus	Penicillin disk	160	25 mm.	Normal culture	454	43	4
	Streptomycin disk	Innumerable	None	Normal culture exposed to streptomycin, 10 units/ml.	366	40	5
	Penicillin residuum exposed to streptomycin, 10 units/ml.	0					
Str. viridans f	Penicillin disk	820	30 mm.				
	Streptomycin disk	Innumerable	None				
	Penicillin residuum exposed to streptomycin, 10 units/ml.	0					
Pneumo. Type 2	Penicillin disk	0	27 mm.				
	Streptomycin disk	Innumerable	None				
Pneumo. Type 9	Penicillin disk	20	32 mm.				
	Streptomycin disk	Innumerable	None				

*Whatman filter paper No. 2; disk 10 mm. in diameter.

Five milliliters of each washing are used for plating, 1 ml. to a plate. One penicillin disk washing is plated with plain agar for residual cell count and another with agar containing streptomycin, 10 units per milliliter, for its effect on penicillin residual organisms. The plates are then incubated overnight. The following morning they are examined; a colony count is made and the number found in the five plates is multiplied by two to get the total number of residual organisms from each paper disk. In order to obtain a ratio of the normal organisms originally in contact with the disk and the remaining viable cells after incubation with the penicillin, one paper disk moistened with broth is placed on a seeded plate and kept in the refrigerator for about one hour to allow the broth in the disk to be absorbed by the agar. The paper disk is then washed in the manner described; the washing is serial diluted and plated for bacterial counts. These are compared with the number of colonies obtained from the penicillin disks. The proportion of killed and surviving bacteria is thus obtained.

It is the belief of some investigators that antibiotics in the same concentration work more effectively on small rather than large numbers of organisms. For this reason we tested small numbers of the test bacteria with the same concentration of streptomycin as was used with the residual cells.

The results of the experiments are summarized in Table IV. While the normal cultures in small as well as in large numbers showed resistance to high concentrations of streptomycin, the residual bacteria were susceptible to low concentrations of the drug, in amounts easily maintained in the body of the patient.

One of the six strains of *Str. viridans* tested did not respond to this treatment. The residual organisms of this strain were not affected by the streptomycin in the concentration used for the other cultures. To show that this method is reliable, two pneumococcus strains, which by the tube method yielded small numbers of residual cells, were also subjected to this test. In one there were no viable cells found on the paper disk. The other yielded twenty colonies, while the zones of inhibition were similar to those which yielded large numbers of viable residual organisms.

DISCUSSION

The results of the foregoing experiments bring to light several important facts in regard to the response of penicillin-sensitive organisms to the action of the drug.

We have shown that penicillin exerts a definite bactericidal effect on all susceptible strains in amounts possible to maintain in the body of the patient. It is capable of actually destroying the great majority of organisms by lysis, or otherwise, under normal cultural conditions. However, there are always some cells left which are capable of withstanding the killing effect of even large doses of penicillin, remaining viable but unable to multiply in its presence. This inhibitory action extends through a wide range of concentrations, so that large and small amounts of the drug have the same inhibitory effect on these residual organisms.

*Whatman filter paper No. 2; disk 10 mm. in diameter.

The tests also indicate a heterogenicity of bacterial cultures in regard to the ability of individual members to react to penicillin. There seem to exist in most bacterial cultures cells of at least three types, differing from each other in the manner in which they react to the drug, namely: (1) Those which are destroyed by lysis; (2) those which are killed without lysis; (3) those which are capable of withstanding the killing action of penicillin, remaining viable but unable to multiply in the presence of the drug. That this threefold response of penicillin sensitive bacteria is not a chance occurrence may be seen from the following findings: (1) When a bacterial culture, on exposure to penicillin, yielded a large number of residual viable organisms it always gave a proportional number of inhibited cells in the cultures for which small inoculum (10^{-4}) was used. (2) In more sensitive strains, in those yielding small numbers of inhibited residual organisms, no viable cells were found in the cultures for which a small inoculum was used (10^{-4}), indicating that, being present in small numbers in the original culture, these cells were eliminated by the dilution. See Table V.

TABLE V. SHOWING PROPORTIONAL NUMBERS OF SURVIVING ORGANISMS IN HIGH CONCENTRATIONS OF PENICILLIN WHEN LARGE AND SMALL INOCULUM WAS USED

BACTERIAL STRAIN	CULTURED IN PENICILLIN (UNITS/ML.)	NUMBER OF ORGANISMS INOCULATED	NUMBER SURVIVED	RATIO OF SURVIVED TO KILLED
Str. viridans a	60	30,000,000	7,000,000	1:4
	60	3,000	724	1:4
Str. viridans b	60	66,000,000	6,000,000	1:11
	60	6,600	500	1:13
Str. viridans c	60	160,000,000	900,000	1:178
	60	16,000	60	1:268
Str. viridans d	60	164,000,000	600,000	1:273
	60	16,400	61	1:270
Staph. citreus	30	100,000,000	110,000	1:910
	30	10,000	9	1:1,100
Staph. aureus	60	95,000,000	7,000	1:13,500
	60	9,500	0	–
Enterococcus	60	11,200,000	6,400	1:1,750
	60	1,120	0	–
Hemolytic streptococcus 98	60	300,000,000	7,000	1:43,000
	60	30,000	0	–
Pneumo. Type 1	20	150,000,000	117	1:1,282,000
	20	15,000	0	–
Pneumo. Type 3	20	105,000,000	128	1:820,000
	20	10,500	0	–
Pneumo. Type 9	20	200,000,000	100	1:2,000,000
	20	20,000	0	–

–, Eliminated by dilution.

It was previously shown by one of us (S. S.)[11] that individual organisms in a bacterial culture, or specimen, also possess different degrees of sensitivity, or resistance, to sulfonamides. However, what makes penicillin a much more potent antibacterial agent is the fact that while the sulfa drugs are only bacteriostatic, penicillin actually destroys the sensitive organisms, leaving, in most cases, only a small number of viable cells capable of withstanding the action of the drug.

We believe that the success of penicillin therapy depends directly on the number of residual organisms left viable after the initial treatment with penicillin. The very small numbers of residual viable organisms in strains of pneumococcus, hemolytic streptococcus, and some strains of staphylococcus, as shown by our tests, may account for the dramatic results so often obtained with penicillin when used in the treatment of acute infections caused by these organisms. On the other hand, the very large numbers of viable organisms found in *Str. viridans* strains, as our tests indicate, may account for the protracted treatment with penicillin necessary in cases of subacute bacterial endocarditis in which *Str. viridans* is the most frequent etiological agent.

With the belief that individual organisms in a given culture possess different inherent characteristics in regard to drug susceptibility, we thought that the residual cells capable of withstanding large amounts of penicillin might perchance be sensitive to the action of other therapeutic agents. We therefore treated bacteria, which remained viable after exposure to penicillin, with streptomycin. The results were striking. While the original parent strain was highly resistant to streptomycin, the residual penicillin-inhibited organisms were destroyed by small amounts of streptomycin. The reason for this apparent discrepancy is probably the fact that the streptomycin-sensitive cells were present in comparatively small numbers in the original culture, so that the action of streptomycin on them was obscured by the overwhelming numbers of resistant cells. Six *Str. viridans* strains were subjected to this treatment and all but one gave similar results, as can be seen from Table IV. While only a small number of bacterial strains were thus tested, we believe the results obtained so far warrant the recording of these findings. If the in vivo action of the antibiotics parallels the action in vitro—and the consensus of opinion is that it does—it is possible that these findings may have a practical application in the treatment of subacute bacterial endocarditis. The administration of large doses of penicillin, if the invader is penicillin sensitive, with interruption of the penicillin treatment once in its early stage by a short course of streptomycin therapy might possibly shorten the course of the disease and lead to permanent clearing of the infection. This would have to be preceded by proper sensitivity tests of both the original infecting organisms as well as the penicillin-residual cells. A simple method for testing penicillin-residual bacteria with streptomycin is included in this paper.

The present methods for testing the susceptibility of bacteria to the action of penicillin are inadequate since their results are not indicative of the true response of the organisms to the drug. By diluting the culture for inoculation, the cells capable of surviving large amounts of penicillin, being present in comparatively small numbers, are eliminated by dilution so that a true cross-section of the bacterial population is not represented. Besides, organisms capable of withstanding high concentrations of penicillin are also inhibited by low concentrations of the drug, so that streaking plates to insure sterility may carry over in the loop sufficient penicillin to inhibit growth on the plate, thus obscuring the actual condition of the cultures. By destroying the penicillin with

penicillinase or by diluting the cultures so that the amount of penicillin left in them is negligible, viable organisms may be recovered, bacterial counts made of the surviving cells, and the size of the residuum of viable bacteria determined. Only in this way may the true condition of a bacterial culture, when exposed to the drug, be determined.

In the method of comparing the titer of sensitivity of a test organism with that of a standard, a hemolytic streptococcus strain is usually employed. The fallacy of this method is discerned when the susceptibility of a hemolytic streptococcus is compared with that of a *Str. viridans* strain. (See Tables II and III.) Both are sensitive to the same degree according to the dilution method of testing. However, when the numbers of residual viable organisms found in each are compared, they can hardly be called equally susceptible to penicillin—23 per cent survivals in *Str. viridans* as compared with 0.05 per cent in the hemolytic streptococcus. The finding of a highly in vitro-sensitive strain of *Str. viridans*, as tested by the present methods, in a case of subacute bacterial endocarditis, which responds poorly to penicillin treatment, becomes less baffling when the true character of such a strain is observed.

SUMMARY

Experiments are presented which show that penicillin is capable of destroying susceptible bacteria, by lysis or otherwise, under normal cultural conditions in amounts possible to maintain in the body of the patient.

The destructive action of penicillin on sensitive bacterial strains, however, is not complete even in high concentrations of the drug. A residuum of viable organisms always remains which is capable of withstanding the destructive action of the antibiotic but is inhibited from multiplying in its presence.

This inhibitory activity of penicillin on the residual viable cells extends through a wide range of concentrations so that large and small amounts of the drug have the same inhibitory effect on these remaining organisms.

The findings suggest that bacterial cultures do not constitute a homogeneous population but that individual members may possess different characteristics as to the manner of reaction under the influence of the antibiotic.

The inferences to be drawn from the results of the experiments in relation to the treatment of infectious diseases with the antibiotics are discussed.

We wish to thank Miss Anne Blevins of the New York Post-Graduate Medical School and Hospital for her kindness in furnishing us with the *Streptococcus viridans* cultures.

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EFFECTS OF THE COLD PRESSOR TEST ON GLOMERULAR FILTRATION AND EFFECTIVE RENAL PLASMA FLOW

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THE cold pressor test is a well-known method of determining the ability of the vasomotor system to respond to reproducible stimuli.[1-4] Exposure of the hand or other body areas to ice water has been shown to cause pain, local vasoconstriction, and an elevation of arterial blood pressure.[5, 6] Recently attention has been drawn to the effects of a local cold stimulus on renal function. Diminished urinary volume, increased specific gravity, decreased urea clearance values, and reduced minute chloride output have been observed following moderately prolonged exposure to the stimulus of the cold pressor test in pregnant and nonpregnant women.[7] Equivocal observations of these phenomena have been reported on other subjects.[8] It is the purpose of this paper to report the effects of local peripheral cold on the specific renal functions of glomerular filtration and effective renal plasma flow.

METHODS

The subjects for these experiments were healthy white male volunteers between the ages of 18 and 37 years who on physical examination and urinalysis showed no evidence of renal disease. The men were asked to abstain from all solid food and liquids, with the exception of one glass of water at bedtime, after supper on the evening prior to the experiment.

On the morning of the experiment the subject assumed a reclining position. An indwelling soft rubber catheter (5.5 mm. in diameter) was installed in the bladder. Following the application of a blood pressure cuff to the left arm, intravenous infusions of isotonic saline were started in the veins of each forearm at a rate of 1 ml. per minute. The needle in the left arm was used for drawing blood samples, that in the right, for the administration of test substances. While these procedures were being carried out (a period of about one hour), the subject ingested 1 liter of water. Then a priming dose of 40 ml. of a 25 per cent mannitol solution* and 3 ml. of a 20 per cent sodium para-aminohippurate solution* was administered intravenously within a period of five minutes. This was followed immediately by a sustaining infusion consisting of a mixture of 600 ml. of isotonic saline, 100 ml. of a 25 per cent solution of mannitol, and 16 ml. of a 20 per cent solution of sodium para-aminohippurate at a rate of 4 ml. per minute. This rate was maintained throughout the experiment.

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*Obtained from Sharp & Dohme, Inc., Philadelphia, Pa.

Zero time was established at thirty minutes after the beginning of the priming dose. Six or seven consecutive clearance periods, of approximately fifteen minutes each, were carried out. After the first two or three of these periods, which served as controls, the subject's left foot was immersed to the level of the malleoli in stirred ice water at 1° C. and was kept there throughout one entire period. Following removal of the foot from the cold stimulus, clearances were measured for three or four more periods.

Approximately five minutes after the beginning of each clearance period, blood samples were drawn through a three-way stopcock attached to the needle in the left arm; care was taken to rinse out the system several times, using withdrawn and reinjected blood in order to wash out any residual saline. Time was noted, to the nearest tenth of a minute, at the beginning and end of the drawing of each sample and the average was taken as the blood sampling time. At the end of each period the bladder was washed with 20 ml. of saline and 20 ml. of air.

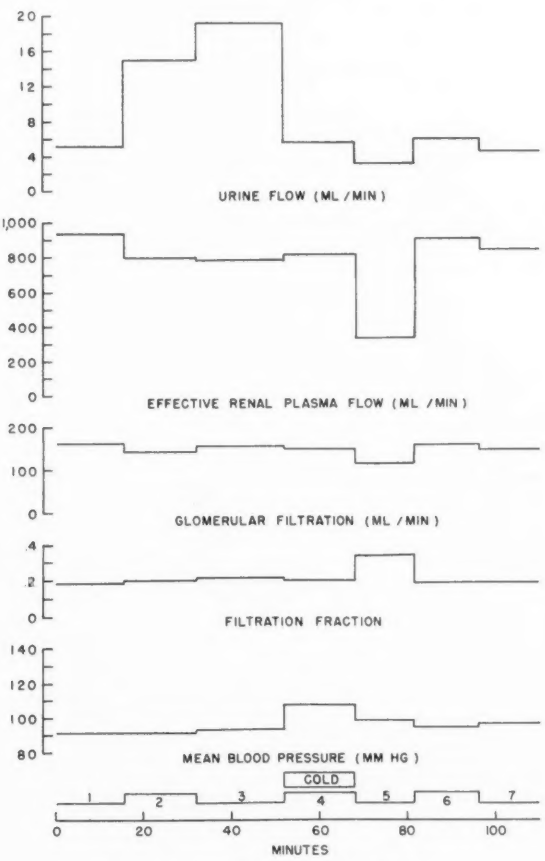

Fig. 1.—Representative experiment.

Auscultatory blood pressures were obtained at least twice during each of the control periods as well as within thirty seconds before and after immersion of the foot in the ice water. During the period of immersion and in the two

TABLE I. SUMMARY OF EXPERIMENTS

SUBJECT	DATE	CONDITION*	NUMBER OF PERIODS	AVERAGE DURATION (MIN.)	URINE FLOW (ML./MIN.)	MANNITOL		PAH		FILTRATION FRACTION† (R)	AVERAGE MEAN B.P.‡ (MM. HG)	CHANGE§ OF C_M‖ (%)	CHANGE§ OF C_{PAH}¶ (%)	CHANGE§ OF AV. MEAN B.P. (%)
						PLASMA (MG./ML.)	CLEARANCE (ML./MIN.)	PLASMA (MG./ML.)	CLEARANCE (ML./MIN.)					
1	9/22/47	a	3	16.6	5.0	.635	164	.0360	711	.23	101			
	9/22/47	c	2	15.3	4.3	.671	150	.0325	542	.28	109	- 9	-24	+ 8
	9/22/47	p	2	14.9	4.8	.687	188	.0252	864	.22	103	+15	+22	+ 2
2	9/23/47	a	3	16.8	13.2	.725	152	.0280	822	.19	95			
	9/23/47	c	2	14.9	4.2	.801	133	.0292	562	.27	107	-12	-32	+13
	9/23/47	p	2	15.5	4.9	.821	147	.0251	843	.18	95	- 3	+ 3	0
3	9/24/47	a	2	15.9	13.1	.463	189	.0233	637	.30	116			
	9/24/47	c	3	14.5	8.7	.495	192	.0190	663	.29	133	+ 2	+ 4	+15
	9/24/47	p	2	15.1	9.4	.641	164	.0188	663	.25	120	-13	+ 4	+ 4
4	9/25/47	a	2	15.0	16.8	.513	182	.0165	696	.26	104			
	9/25/47	c	2	15.0	6.4	.519	132	.0141	508	.26	117	-28	-27	+13
	9/25/47	p	2	14.8	6.9	.546	154	.0178	645	.24	106	-15	- 7	+ 2
5	9/29/47	a	2	15.0	23.3	.744	153	.0193	926	.17	106			
	9/29/47	c	1	15.3	15.3	.770	118	.0180	711	.17	124	-23	-23	+17
	9/29/47	c	2	14.8	5.4	.936	117	.0192	737	.16	124	-23	-23	+17
	9/29/47	p	2	12.5	5.7	.972	128	.0170	862	.15	115	-16	- 7	+ 8
6	9/30/47	a	2	14.8	12.2	.502	179	.0150	891	.20	90			
	9/30/47	c	2	15.1	5.2	.590	154	.0175	566	.27	108	-14	-26	+20
	9/30/47	p	2	15.3	5.7	.680	191	.0160	790	.25	89	+ 7	+ 7	- 1
7	10/ 6/47	a	2	15.9	11.3	.791	116	.0244	627	.19	95			
	10/ 6/47	c	3	15.2	2.7	.810	102	.0256	486	.21	106	-12	-22	+12
	10/ 6/47	p	2	16.0	3.1	.971	114	.0266	655	.17	98	- 2	+ 5	+ 3
Average % of control		c										-14	-21	+14
Average % of control		p										- 4	+ 4	+ 3

*a, Control periods; c, periods of cold effect; p, recovery periods.

†Filtration fraction $= \frac{\text{Mannitol clearance}}{\text{PAH Clearance}}$

‡(Systolic + diastolic) ÷ 2 = Mean B.P. average of all readings.

§Change from control (a) average.

‖C_M, mannitol clearance.

¶C_{PAH}, Para-aminohippurate clearance change from control (a) average.

following periods determinations were made at approximately two-minute intervals. Thereafter, the frequency of the readings was similar to that during the control periods.

Analyses for mannitol and sodium para-aminohippurate were carried out on heparinized plasma and on diluted urine samples according to the methods of Corcoran and Page[9] and Smith and co-workers,[10] respectively. It has been suggested that the clearance of mannitol may be slightly lower than the true glomerular filtration rate.[11] This would not alter the interpretation of these experiments since the importance of these data lies in their relative rather than in their absolute values.

RESULTS

The results of these experiments are summarized in Table I. A representative experiment is shown in Fig. 1. As will be noted, in six out of seven subjects both glomerular filtration rate and effective renal plasma flow decreased either during the application of the cold stimulus or within approximately thirty minutes thereafter. In no subject did the effect persist longer. The average decreases in glomerular filtration rate and effective renal plasma flow, as compared with the controls, were 14 per cent and 21 per cent, respectively. The observed depression of urine flow confirms the finding of Odell and Aragon.[7]

In all subjects the blood pressure rose promptly after application of the cold stimulus and this rise was sustained throughout the period of immersion. Upon removal of the stimulus, the blood pressure gradually decreased, returning to control levels in fifteen to twenty minutes. Examination of these data reveals no correlation between the degree of blood pressure elevation and the observed changes in renal function.

DISCUSSION

The application of a peripheral cold stimulus is found to decrease urinary minute volume, glomerular filtration rate, and effective renal plasma flow.

The large reduction of urine flow as compared with the moderate depression of glomerular filtration rate is regarded as evidence of alteration in the tubular reabsorption of water. This antidiuretic response may be of the same nature as that demonstrated by Rydin and Verney in dogs subjected to emotional stress.[12]

The results obtained on Subject 3 (26 years of age) may be of interest. While the blood pressure rose in all experiments as a result of the stimulus, the rise was not associated with changes in glomerular filtration and renal plasma flow in this one subject. The initial diastolic blood pressure (128/90) together with the blood pressure response (164/116), during exposure to cold, would suggest, according to the criteria of Hines and Brown,[13] that this individual belongs to the prehypertensive group. However, as indicated above, these observations do not establish a correlation between the degree of blood pressure rise and the changes in renal function in response to the cold stimulus.

The mechanisms ultimately responsible for these findings remain to be identified and are being investigated.

change from control (a) average.
(Cm, mannitol clearance.
(Cpah, Para-aminohippurate clearance change from control (a) average.

SUMMARY

Studies were made of the effects of the cold pressor test on renal function. Seven male volunteers who had no history of renal disease served as subjects. Glomerular filtration (as measured by mannitol clearance) and effective renal plasma flow (as measured by sodium para-aminohippurate clearance) were determined before, during, and after immersion of the foot in ice water at 1° C. for fifteen minutes.

In six out of seven subjects both glomerular filtration rate and effective renal plasma flow decreased either during the application of the cold stimulus or within approximately thirty minutes thereafter. In no subject did the effect persist longer. The average decreases in glomerular filtration rate and effective renal plasma flow, as compared with the control values, were 14 per cent and 21 per cent, respectively.

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RELATION BETWEEN STRUCTURAL AND FUNCTIONAL ALTERATIONS OF THE LIVER

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A CORRELATION between basic histopathologic phenomena in the liver and the results of the different liver function tests is still not established. In the past, repeated attempts to secure such a correlation were primarily based upon animal investigations with experimentally produced well-defined conditions.[1-4] In the human being, several approaches were attempted. One approach was the comparison between the histologic picture found in autopsy material and the results of liver function tests carried out shortly before death. This is objectionable because of the marked changes which occur during the agonal period.[5] Another approach was the performance of serial function tests during the course of a disease with well-established histologic pictures as for example, infectious hepatitis or obstructive jaundice.[6-9] Only recently has a systematic attempt been made to compare the results of function tests with the histologic picture seen in biopsies.[10-12] In the following study, utilizing a relatively large series of cases of different diseases, a statistical attempt has been made to compare morphologic phenomena, independent of the underlying disease, with the results of liver function tests carried out at the time of the biopsy. The utilization of a larger material appeared desirable to overcome the obvious overlapping caused by the occurrence of more than one of the basic histologic phenomenon in a given case.

A statistical correlation between morphologic alterations and the results of liver function tests was carried out to study the following two problems:

1. Evaluation of different liver function tests by comparison of their results with the presence and degree of liver cell damage.
2. Functional significance of the different basic morphologic phenomena.

MATERIAL AND METHODS

Patients suffering from various stages of different liver diseases or hepatomegalies make up the material of this study. The liver diseases studied include acute infectious and toxic hepatitis, extrahepatic biliary obstruction due to tumor or stone, different types of cirrhosis, and a miscellaneous group which included such conditions as amyloidosis, Boeck's sarcoid, lymphosarcoma, xanthomatosis, and so on. A total of one hundred sixty-five biopsies which included thirty-five repeat biopsies on the same patients was performed and the histologic findings were statistically correlated with the results of a series of function tests carried out within two days of the biopsy. All function tests were not carried out in every case. The biopsies in the great majority of cases were performed by means of the Turkel needle,[13] utilizing the lateral approach through the seventh to tenth right costal interspace. In a few

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instances they were performed during laparotomy. The biopsy material was fixed in Zenker-formalin or Carnoy's solution. Sections were stained with hematoxylin-eosin, Mallory's aniline blue connective tissue stain, and Gömöri's reticulum fiber stain. The following histopathologic phenomena were correlated with results of liver function tests: (1) diffuse liver cell damage, (2) focal necrosis, (3) regeneration, (4) distorted reconstruction, (5) periportal inflammatory activity, (6) fatty metamorphosis, and (7) increased Kupffer cell activity.

A tetrachoric coefficient[14, 15] of correlation of these phenomena with results of the biochemical determination was chosen for statistical correlation. By this method, one is enabled to correlate the presence or absence of one variable (histopathologic phenomenon) with the presence or absence of a second variable (abnormal result of function test). This coefficient, when corrected for the number of cases, yielded a critical ratio (CR). This mathematical figure (CR) indicates the degree to which the obtained results represent a reliable or consistent trend. A critical ratio above 2 is considered significant. The formula employed and an example in the derivation of a CR value follows.

$$CR = \frac{ad - bc}{\sqrt{(a+b)(c+d)(a+c)(b+d)}} \div \frac{1}{\sqrt{N}}$$

Variable 2	Variable 1 +	Variable 1 −	
+	a	b	a + b
−	c	d	c + d
	a + c	b + d	N

Focal Necrosis	Sedimentation Rate: Abnormal	Normal	Total
Present	53	37	90
Absent	24	20	44
	77	57	134

$$CR = \frac{1060 - 888}{\sqrt{16{,}984{,}440}} \div \frac{1}{\sqrt{134}} = +0.482$$

For use in scatter graphs the pathologic phenomena were subjectively graded from 1 plus to 4 plus.

Limits between normal and pathologic results of each of the applied function tests were arbitrarily drawn (Table I). In general, the borderline of the pathologic levels chosen is the one currently accepted. In some instances, however, the borderline has been set higher than that usually employed since only the markedly pathologic levels were expected to show a correlation. This was especially true in the case of total serum bilirubin and also cholesterol ester ratio, prothrombin time, and sedimentation rate. For serum alkaline phosphatase two levels were selected: 4 to 10 Bodansky units as found as a rule in liver cell damage, and

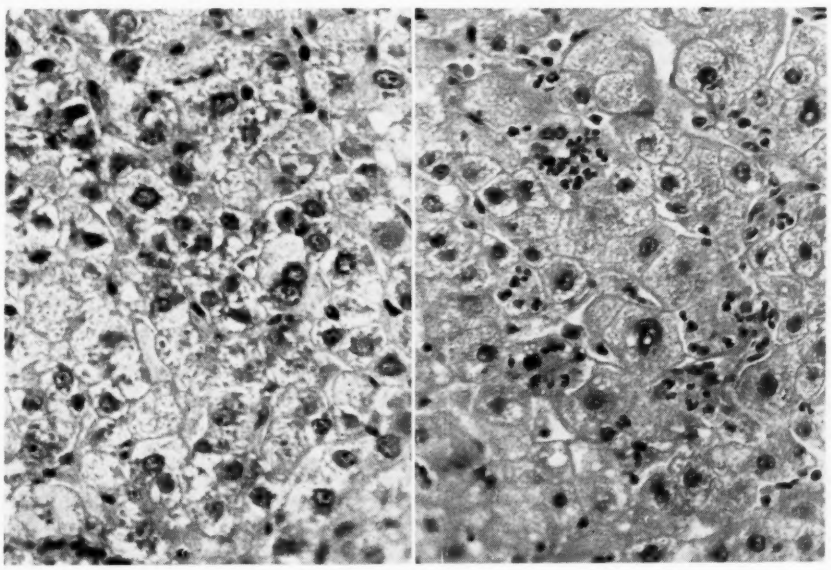
TABLE I. FUNCTION TESTS AND LEVELS ARBITRARILY SELECTED FOR CORRELATION WITH HISTOPATHOLOGIC PHENOMENA

Serum total protein	Below 6 Gm. %
Albumin/globulin ratio	Below 1.0
Serum nonprotein nitrogen	Above 35 mg. %
Cephalin cholesterol flocculation	+++ and ++++[16]
Thymol turbidity	Above 4 units[17, 18]
Total serum cholesterol	Above 250 mg. %
Percentage of cholesterol ester in total serum cholesterol	Below 45%[19, 20]
Serum alkaline phosphatase	Above 10 BU[21]
Total serum bilirubin	Above 8 mg. %[22]
Bromsulfalein retention (5 mg./kg. in 45 min.)	Above 10%
Urinary urobilinogen	Above 3 Ehrlich units[23]
Stool urobilinogen	Below 10 Ehrlich units[23]
Prothrombin time	Below 80%[24]
Serum vitamin A	Below 15 μg[25]
Sedimentation rate	Above 30 mm./hr.

above 10 units as found usually in extrahepatic biliary obstruction. Decreased pathologic values of urinary urobilinogen were not utilized in this study. However, a reduction of stool urobilinogen to below 10 units was considered evidence of biliary obstruction and was used in the statistical correlation. The level selected for serum vitamin A was quite low. Abnormal elevation of vitamin A was not found in this series.

RESULTS

Diffuse liver cell damage (Fig. 1, *A*) was considered to be present when all or almost all liver cells revealed one or more of the following aberrations from the normal: dissociation of the liver cell cords, unusual variation in size of the cells, hazy borders, irregular staining of the cytoplasm with appearance of irregularly shaped vacuoles (apparently not containing fat), clumping of cytoplasm with marked basophilia of the clumped material, eosinophilic coagulation necrosis, irregular size, shape, and staining of the nuclei leading to pyknosis



A. *B.*

Fig. 1.—Photomicrographs of liver biopsy specimens. *A*, Diffuse liver cell damage in a case of infectious hepatitis. Cytoplasm and nuclei of the liver cells vary in size and staining qualities. The outline of the cells is hazy and the architecture of the cords is irregular. *B*, Focal necrosis in a case of cholecystitis. The liver cells have a normal appearance. In small areas they are necrotic and are replaced by polymorphonuclear leucocytes which accumulate in the perisinusoidal spaces.

or ballooning of the nuclei ("glycogen" nuclei), and hyalinization of the entire cell. These changes varied in intensity throughout the lobule but some degree of damage was present in the entire lobule.

As seen from Table II, there was a statistically significant correlation between liver cell damage and cephalin-cholesterol flocculation, thymol turbidity, albumin/globulin ratio, and bromsulfalein retention. A less significant relation was found to marked decrease in prothrombin, high values of serum bilirubin and slightly elevated levels of alkaline phosphatase (4 to 10 Bodansky units). Levels of alkaline phosphatase above 10 units showed little correlation.

TABLE II. CORRELATION BETWEEN MORPHOLOGIC PHENOMENA AND PATHOLOGIC RESULTS OF LIVER FUNCTION TESTS AS EXPRESSED BY THE CRITICAL RATIO (A RATIO ABOVE +2 INDICATES A SIGNIFICANT RELATION)

	LIVER CELL DAMAGE	FOCAL NECROSIS	REGENERATION	DISTORTED RECONSTRUCTION	PERIPORTAL ACTIVITY	FATTY CHANGES	KUPFFER CELL ACTIVITY
Total protein	−2.64	−3.07	−1.99	−0.82	−0.43	+0.56	−0.50
A/G ratio	*+4.19*	+0.43	+0.28	+0.81	+0.78	−1.65	*+4.57*
Nonprotein nitrogen	+1.23	−1.91	+0.61	−1.32	−2.54	−1.40	−0.48
Cephalin cholesterol flocculation	*+4.39*	+1.53	−0.56	*+4.47*	+1.92	+1.68	−0.85
Thymol turbidity	*+5.15*	+0.14	*+2.87*	*+2.97*	+0.31	+1.27	+1.92
Total cholesterol	−1.03	+1.36	+1.36	−2.81	−2.15	+0.52	+1.48
Cholesterol esters	+0.59	−2.39	+0.21	+0.50	+1.27	+0.80	+0.07
Alkaline phosphatase (as a whole)	+0.27	−0.27	+0.60	+0.75	−3.17	−0.96	−0.19
Alkaline phosphatase (4-10 BU)	*+2.54*						
Alkaline phosphatase (above 10 BU)	+0.05						
Serum bilirubin	*+2.52*	−2.36	+1.16	−1.67	+0.38	−2.41	*+3.54*
Bromsulfalein	*+3.92*	−0.90	+0.52	−0.87	+0.50	−0.19	+0.08
Urinary urobilinogen	+0.98	−1.42	−2.65	+1.83	−0.85	−0.97	−0.71
Fecal urobilinogen	−0.12	−2.48	+1.41	−1.17	−1.71	+0.12	−0.44
Prothrombin time	*+2.09*	−2.23	−1.88	+0.62	+1.42	−0.16	−0.05
Plasma vitamin A	*+2.67*	−0.36	+1.42	+1.14	+0.67	−0.28	+0.83
Sedimentation rate	+0.02	+0.48	+0.16	*+2.77*	*+2.95*	−0.51	−4.63

as did the results of the alkaline phosphatase when taken as a whole. There was no relation between histologically recognizable parenchymal damage and abnormal levels of total protein, urinary urobilinogen, decrease in cholesterol ester ratio, elevated nonprotein nitrogen, and sedimentation rate.

Among the scatter graphs which plot the quantitative relation between liver cell damage and the results of various liver function tests, three appear particularly instructive. No quantitative relation was found between parenchymal damage and total serum protein levels (Fig. 2), except at very low values (cases of hypoproteinemia due to causes other than liver disease were not included in this study). The degree of morphologic damage associated with total protein levels between 5 and 8 per cent was similar. However, there was an inverse quantitative relation between the albumin/globulin ratio and the degree of parenchymal damage (Fig. 3). The relation between liver cell damage and cephalin-cholesterol flocculation is more complicated (Fig. 4). The plotted (composite) curve shows that the degree of liver cell damage is proportional to the cephalin-cholesterol flocculation between the ranges of 1 plus and 4 plus. However, a greater degree of damage coincided with cephalin-cholesterol flocculation values of 0 than those of 1 plus. The explanation for this may lie in the fact that many patients with negative cephalin-cholesterol flocculation had extrahepatic biliary obstruction secondary to tumor or lithiasis and some of these morphologically exhibited marked liver cell damage. On the other hand, most of the cases in the 1 plus to 4 plus range had primary hepatitis or cirrhosis.

Focal necrosis (Fig. 1, *B*) was indicated by small, irregularly scattered areas in which the parenchymal liver cells were either absent or present in the form of small anuclear fragments. They were replaced by large numbers of round

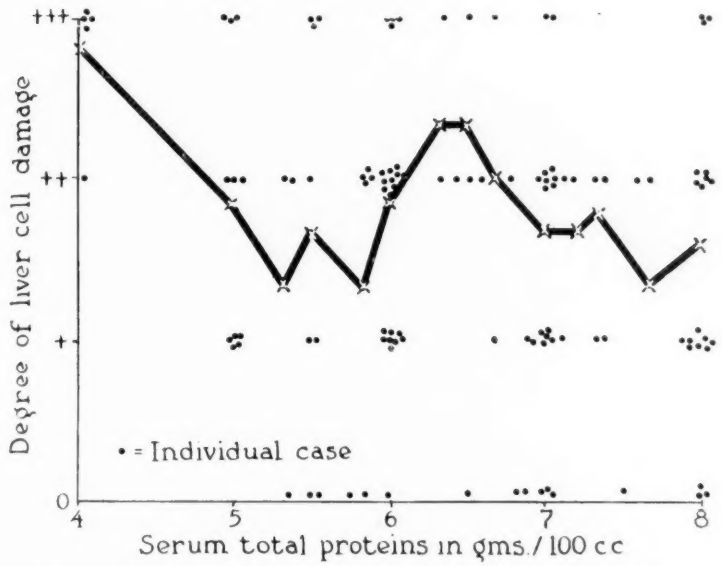


Fig. 2.—Relation between serum total proteins and liver cell damage.

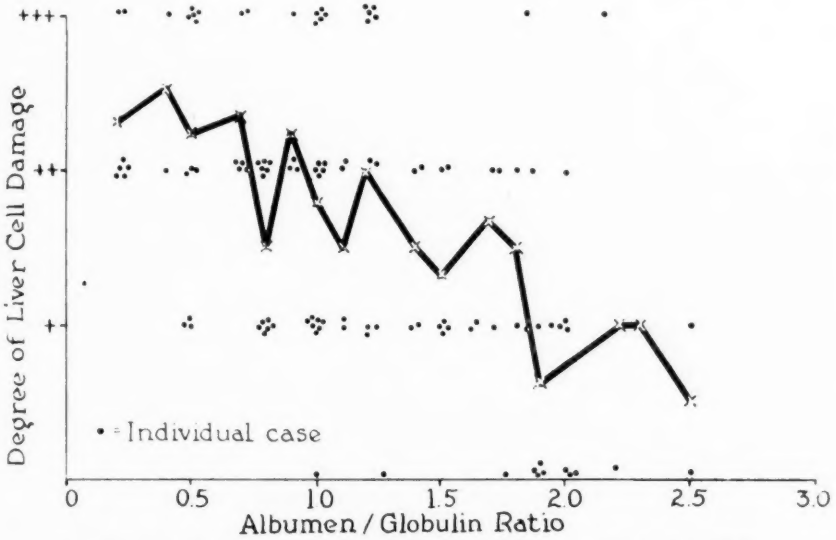


Fig. 3.—Relation between albumin/globulin ratio and liver cell damage.

cells or polymorphonuclear leucocytes, giving these areas a rather cellular appearance. No definite correlation between focal necrosis and any of the function tests was found.

Regeneration of liver cells (Fig. 5, *A*) was characterized by large parenchymal cells often containing more than two nuclei which were frequently bizarrely shaped. These regenerating cells were found either in protracted liver cell damage or in apparent recovery from a preceding injury. This morphologic phenomenon showed a correlation only with elevated levels of the thymol turbidity test.

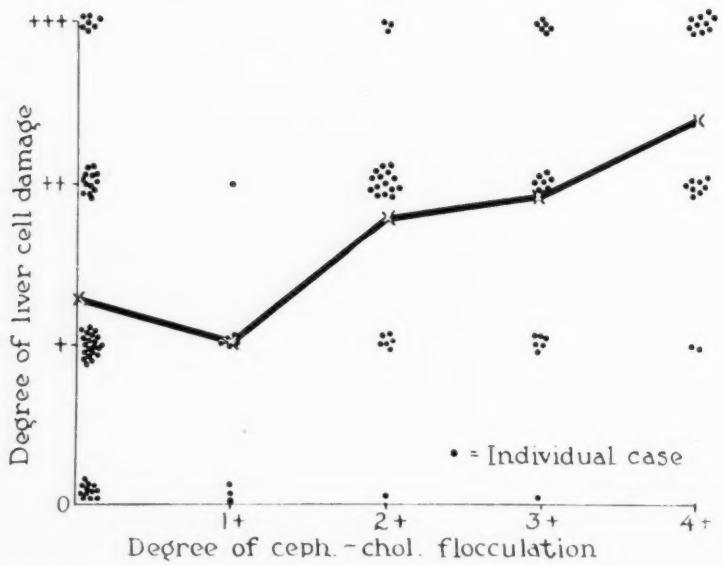
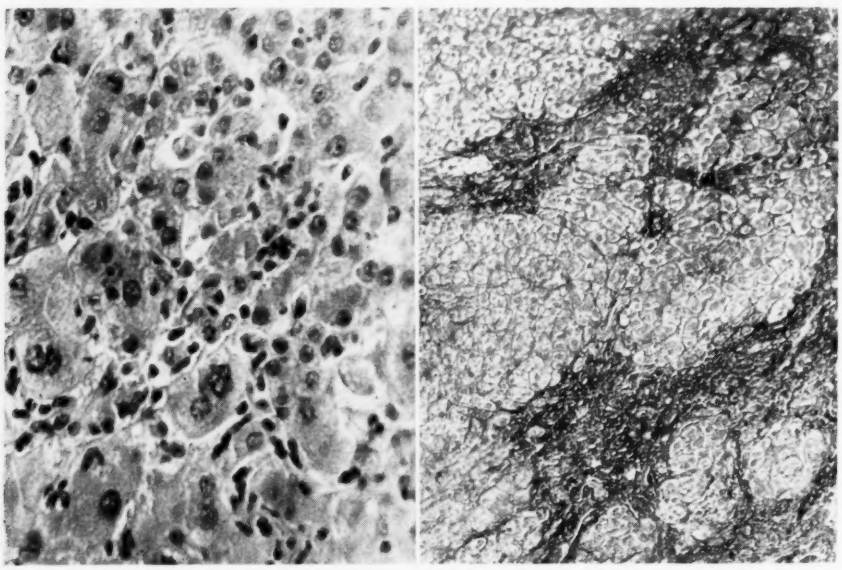


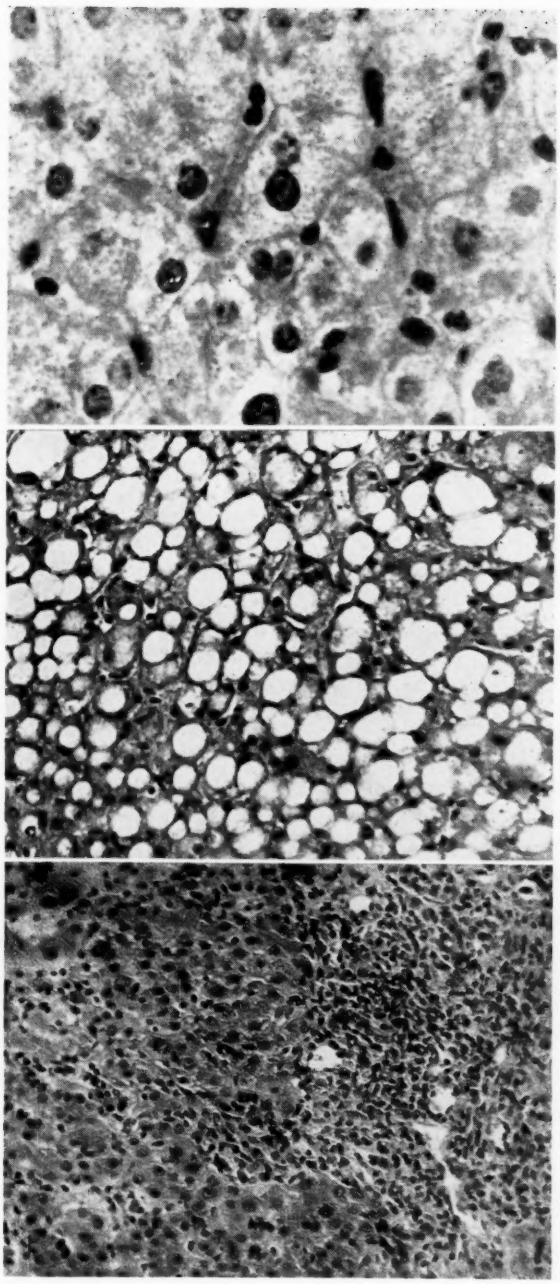
Fig. 4.—Relation between cephalin-cholesterol flocculation and liver cell damage.



A. *B.*

Fig. 5.—Photomicrographs of liver biopsy specimens. *A*, Marked liver cell regeneration in a case of chronic infectious hepatitis. The liver cells in general reveal little damage but some of them are large and have two or three nuclei and abundant cytoplasm. There is some proliferation of histiocytic cells. *B*, Distorted reconstruction in a case of portal cirrhosis (Mallory's aniline blue stain). Small nodules devoid of the usual lobular architecture are separated by wide connective tissue trabeculae.

Distorted reconstruction of the liver parenchyma (Fig. 5, *B*) denoted partial or complete loss of the lobular pattern with replacement by parenchymal nodules, the cells of which were not arranged around a central vein. These pseudolobules varied in size, were often found near the periportal field, and were usually well demarcated by more or less dense connective tissue trabeculae.



A. *B.* *C.*

Fig. 6.—Photomicrographs of liver biopsy specimens. *A*, Periportal inflammatory activity. There is dense lymphocytic and histiocytic infiltration of the widened periportal field. The inflammatory exudate is arranged around lymphatics. There is some proliferation of the septal bile ducts. *B*, Fatty metamorphosis in a case of acute toxic hepatitis. Large fat droplets replace the cytoplasm of most of the liver cells. *C*, Kupffer cell mobilization (high power). The liver cells reveal normal appearance. The Kupffer cells are separated from the liver cell cords. They are large and bulge forward into the sinusoids.

This phenomenon occurred in cirrhosis. Distorted reconstruction revealed significant correlation with cephalin-cholesterol flocculation and a less significant one with thymol turbidity and sedimentation rate.

Periportal inflammatory reaction (Fig. 6, *A*) described inflammatory changes in the portal triads usually originating in and around the lymphatics. These changes were characterized by accumulations of round cells and only rarely of polymorphonuclear leucocytes. The cellular accumulations varied in size and shape. Proliferation of septal bile ducts was frequently associated with the more marked of these inflammatory changes. The reaction showed a significant relation to increased sedimentation rate and only a minor one to the cephalin-cholesterol flocculation test.

Fatty metamorphosis (Fig. 6, *B*) was characterized by accumulation of fat in the liver cells in the form of various sized droplets. There appeared to be no significant relation between these fatty changes and any of the function tests.

Increased Kupffer cell activity (Fig. 6, *C*) denoted their mobilization and proliferation. This was characterized by an increase in the number and an enlargement of the individual Kupffer cells. They were separated from the liver cell cords and their abundant cytoplasm extended into the lumen of the sinuses and often contained phagocytosed material. This picture showed a significant correlation with the albumin/globulin ratio and with a marked increase in serum bilirubin.

COMMENT

In the interpretation of the correlations found, it must be kept in mind that they are statistical, and association must not be confused with causation. The fact that a morphologic phenomenon may be associated with abnormal values of a certain function test is no proof that the abnormal function is caused by that pathologic change. Some common factor might be responsible for both morphologic change and functional alteration.

As expected, diffuse liver cell damage revealed, in general, a good correlation with many of the liver function tests. In some instances, however, liver function tests were negative in the presence of visible liver cell damage. Moreover, in individual cases there were various combinations of positive liver function tests. The pattern of these variations is only partly known. Thus the cephalin-cholesterol flocculation test was almost invariably negative in uncomplicated extrahepatic biliary obstruction.

Focal changes, though morphologically often far more impressive than generalized liver cell damage (the recognition of which is not always a simple matter), are not necessarily associated with liver function impairment. The bulk of the still intact liver parenchyma compensates for the relatively few cells which are destroyed. This observation is well in keeping with the widespread occurrence of focal necrosis in a multitude of diseases in which no functional liver impairment is found. Hence, conclusions concerning the extent of liver cell damage should not be drawn from isolated though anatomically impressive alterations.

Fatty changes as such do not interfere markedly with liver function. Distorted reconstruction as an expression of cirrhosis is related to some but not to all of the function tests. Except for the thymol turbidity test there is no statistically recognizable relation between regenerative processes and any of the function tests. The increased Kupffer cell activity in cases of severe jaundice is probably best explained by the large amounts of bile pigment in them. The inflammatory nature of the portal reaction explains its correlation with the elevation of the sedimentation rate; however, no relation to other function tests was demonstrated.

The evaluation of the various function tests by a morphologic method rendered, in general, results similar to those of other methods.[26-28] In contrast, however, to the findings of Sherlock,[10] this method failed to reveal a significant correlation between total serum protein concentration and any of the examined pathologic phenomena. The albumin/globulin ratio appeared to be a much better index of liver cell damage. The total protein concentration without albumin-globulin partition is no indicator of liver cell damage except when markedly decreased. With marked liver cell damage, the albumin/globulin ratio may be reversed with normal total protein values. This is partly explained by reduced formation of albumin by the damaged liver. The demonstrated correlation between Kupffer cell mobilization and the albumin/globulin ratio is probably due to hyperglobulinemia which is known to occur with stimulation of the reticuloendothelial system.[29] The nonprotein nitrogen appears to be unrelated to any of the examined phenomena. Elevation of nonprotein nitrogen and urea nitrogen in liver disease (in some forms of hepatitis and in prolonged extrahepatic biliary obstruction) is primarily a renal phenomenon due to pathologic reabsorption of urea in the renal tubules.[30, 31]

The cephalin-cholesterol flocculation revealed a good correlation with liver cell damage, since in the absence of parenchymal damage the cephalin flocculation is almost invariably negative. This observation, obtained by statistical evaluation of the entire material, must be qualified when individual cases are analyzed. The cephalin-cholesterol flocculation is usually negative in uncomplicated extrahepatic biliary obstruction, even if severe liver damage is visible under the microscope. With equal degrees of morphologically demonstrable liver damage, the cephalin-cholesterol flocculation is pathologic in primary hepatitis (infectious or toxic) or in cirrhosis and normal in an uncomplicated biliary obstruction due to tumor or stone. If the biliary obstruction is complicated by bacterial infection of the portal triads, the cephalin-cholesterol flocculation becomes positive.[12] The aforementioned characteristics of the cephalin-cholesterol flocculation test make it especially useful from a practical diagnostic standpoint.[32-35] No explanation can be offered for the fact that a test which depends on the relation between albumin and gamma globulin was negative in prolonged uncomplicated biliary obstruction despite advanced liver cell damage. The fact that this test is usually positive in cirrhosis accounts for its close correlation with distorted reconstruction of the lobular pattern.

The thymol turbidity reveals statistically the most significant correlation with liver cell damage. The close correlation with regeneration agrees with

observations that this test remains positive in infectious hepatitis longer than the other tests. Based on this, Kunkel and Hoagland[36] associated the thymol turbidity with regeneration. Neefe,[37] however, connected it with periportal infiltration, with which we found no correlation. Whether the thymol turbidity is an expression of regeneration also in the early stages of infectious hepatitis is as yet unsettled. Its relation to reconstruction can be interpreted similarly to that of the cephalin-cholesterol flocculation test.

The total serum cholesterol and cholesterol ester fraction showed no significant correlation with any of the examined morphologic alterations. The total cholesterol elevation in surgical jaundice is probably due to obstructed bile flow. Cholesterol ester reduction in the material studied appeared too erratic to be statistically significant.

Elevation of serum alkaline phosphatase in general showed no relation to the studied morphologic changes. However, if the results of the alkaline phosphatase tests are broken down, the group between 4 and 10 Bodansky units revealed a relation to liver cell damage but not the group above 10 Bodansky units. Marked elevation of the alkaline phosphatase level is considered primarily the result of retention of the enzyme due to interference with biliary excretion,[33] since it occurs chiefly in cases of surgical jaundice; this accounts for the absence of relation to liver cell damage. In the group with values between 4 and 10 units, medical jaundice predominated. This level might be explained by a lesser degree of bile flow interference or, as has been claimed, by an increased formation by the damaged liver cells.[38-41]

Bromsulfalein retention (studied only in nonjaundiced patients) was related only to liver cell damage. Elevation of urinary urobilinogen was usually absent without liver damage. On the whole, however, in the applied approach, they could not be correlated with each other since in the presence of biliary obstruction (intra- or extrahepatic) urobilinogen may be absent from the urine despite marked liver cell damage. Stool urobilinogen, being primarily an indicator of biliary excretion, showed no relation to the studied phenomena.

The close relation of reduction of prothrombin to liver cell damage is due to the fact that in the absence of parenchymal damage the prothrombin time was invariably normal. However, the reverse did not hold true, that is, the prothrombin time was occasionally normal in spite of histologically recognizable cell damage. As generally accepted, a pathologic prothrombin time is evidence of liver function impairment, provided other causes of hypoprothrombinemia such as poor vitamin K absorption due to obstructed bile flow or intestinal disorders are ruled out.

The reduction of the plasma vitamin A level showed a fair relation to liver cell damage in that when low values were found in liver disease the parenchymal cells almost constantly appeared damaged. Low vitamin A levels have been considered a test for impaired hepatic function.[25, 42, 43] Obviously, the reduction can also be caused by other factors leading to endogenous vitamin A deficiency. This explains why in some instances without liver cell damage low vitamin A values were found.

The sedimentation rate revealed a correlation with periportal activity, probably because of the latter's inflammatory character. The correlation of the sedimentation rate with distorted reconstruction of the lobular pattern may stem from the fact that cases of active cirrhosis showing such reconstruction usually exhibit inflammatory periportal activity.

In general, the presented correlations may aid in the differential diagnosis of liver disease by a morphological evaluation of the function. An association between function tests and morphologic alterations, even without causative connection, is significant.

SUMMARY

In one hundred thirty patients (thirty-five repeatedly studied) suffering from various liver diseases, the histologic picture of the liver as seen in biopsy specimens obtained by aspiration or laparotomy was compared with the results of a series of liver function tests performed at the time of biopsy.

The incidence of seven morphologic phenomena (diffuse liver cell damage, focal necrosis, regeneration, distorted reconstruction, periportal activity, fatty metamorphosis, and Kupffer cell mobilization) was compared statistically with the results of each of different liver function tests.

A significant correlation was found between diffuse liver cell damage and albumin/globulin ratio, cephalin-cholesterol flocculation, thymol turbidity, and bromsulfalein retention; a lesser degree with highly elevated serum bilirubin, reduced plasma vitamin A, increased prothrombin time, and slightly elevated alkaline phosphatase. No correlations were elicited between parenchymal damage and total serum protein, total cholesterol, cholesterol ester ratio, urinary urobilinogen, stool urobilinogen, alkaline phosphatase in general (and markedly elevated alkaline phosphatase specifically), nonprotein nitrogen, or sedimentation rate.

Focal necrosis, in contrast to diffuse liver cell damage, was not associated with significant changes in liver function. The same was found with fatty metamorphosis.

Regeneration showed a correlation with increased thymol turbidity. Distorted reconstruction of the lobular pattern (as seen in cirrhosis) was related to cephalin-cholesterol flocculation, thymol turbidity, and elevation of sedimentation rate. Kupffer cell activity appeared related to elevated serum bilirubin and pathologic albumin/globulin ratio.

These findings provided a basis for the morphologic evaluation of various liver function tests and a discussion of the functional significance of the aforementioned pathologic phenomena.

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THE IMPORTANCE OF THE RATE OF DYE REMOVAL IN THE BROMSULFALEIN TEST OF LIVER FUNCTION

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FOLLOWING the introduction of bromsulfalein (BSP) retention as a test of liver function by Rosenthal and White[1] in 1925, numerous attempts have been made to improve the clinical method. Currently, the accepted procedure for the test is that recommended by Mateer and co-workers.[2] This procedure uses intravenously 5 mg. of BSP per kilogram body weight and accepts 4 per cent retention in forty-five minutes as the upper limit of normal liver function. The BSP concentration in serum is determined by the method of Gaebler.[3]

Several years ago MacDonald[4] published clinical evidence indicating the value of serial BSP determinations in the detection of liver damage. By taking blood samples at frequent intervals after the injection of 5 mg. per kilogram of BSP intravenously, he was able to detect liver damage not evident from a single forty-five minute blood sample. The study here reported was undertaken to compare the value of the fifteen-minute blood sample with the forty-five minute sample in the detection of liver damage.

EXPERIMENTAL OBSERVATIONS

There is abundant evidence that intravenously injected BSP is not quantitatively excreted in the bile, but is eliminated by other systems of the body, particularly the reticulo-endothelial system.[5, 6, 7, 8] After determining in preliminary experiments in dogs that 5, 10, and 20 mg. per kilogram of BSP intravenously yielded reproducible and quantitatively similar curves, the effect of blocking the reticulo-endothelial system with India ink was studied. Fig. 1 records a typical experiment. It will be noted that, although within 48 hours after the injection of the India ink the forty-five minute serum BSP level was below 1 mg. per cent (10 per cent retention), as long as 192 hours later the fifteen-minute serum concentration remained above the control level.

Following splenectomy (Fig. 2) with removal of this portion of the reticulo-endothelial system, there was a temporary increase in BSP retention. As illustrated in Fig. 2, this retention was evident in the fifteen-minute serum sample two days after the forty-five minute sample had returned to control levels.

After obtaining a control BSP retention test using 5 mg. per kilogram intravenously, hepatic damage was produced in rabbits by giving 0.5 ml. per kilogram of carbon tetrachloride in corn oil via stomach tube. BSP retention was tested two days and ten days after the administration of carbon tetrachloride. Table I records the marked BSP retention that occurred in the fifteen-minute serum sample long after the forty-five minute sample had returned to control levels.

The results of these experiments indicated that, when the reticulo-endothelial system was compromised (India ink injection, splenectomy) or when

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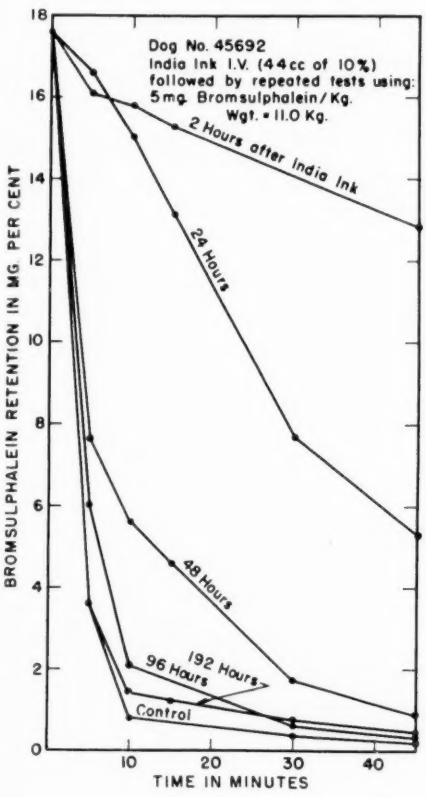

Fig. 1.

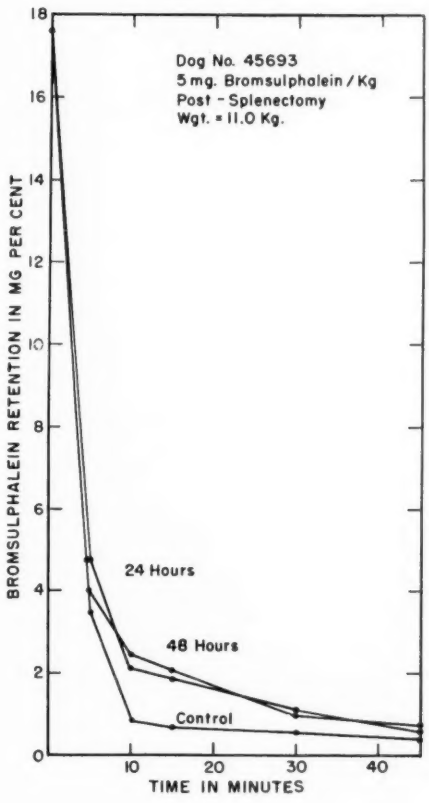

Fig. 2.

TABLE I. PERCENTAGE OF BROMSULFALEIN RETENTION

RABBIT NO.	CONTROL		TWO DAYS AFTER CCl_4		TEN DAYS AFTER CCl_4	
	15 MIN.	45 MIN.	15 MIN.	45 MIN.	15 MIN.	45 MIN.
59607	3	1	65	4	15	2
59549	1	0	71	3	9	1
59603	3	0	58	2	6	1
59606	4	1	54	2	45	12

hepatic change was produced by the oral administration of carbon tetrachloride, the fifteen-minute serum BSP sample reflected the change long after the forty-five minute sample had become normal.

CLINICAL OBSERVATIONS

BSP determinations using Gaebler's method[3] and 5 mg. per kilogram doses in twenty individuals with normal liver function are recorded in Table II. Table III includes the BSP retention data from twenty-one individuals hospitalized with miscellaneous disorders.

All the normal individuals (Table I) had a fifteen-minute BSP serum concentration below 25 per cent. This finding agrees with the observations of MacDonald,[4] who reported more than 25 per cent retention in fifteen minutes in only three of thirty-eight patients. In Patients 1, 7, 8, 9, and 21 in Table III

TABLE II. BROMSULFALEIN RETENTION IN INDIVIDUALS WITH NORMAL LIVER FUNCTION

PATIENT	DIAGNOSIS	SEX	AGE (YR.)	BROMSULFALEIN RETENTION (%)	
				15 MIN.	45 MIN.
1	Upper respiratory infection	M	54	14	3
2	Multiple sclerosis	F	34	5	0
3	Hypothyroidism	F	38	22	3
4	Contusions of face	F	52	19	4
5	Pneumonia	M	54	14	3
6	Morphine addiction	M	54	20	4
7	C.N.S. syphilis	M	55	5	2
8	Hypothyroidism	F	30	5	1
9	Pneumonia	M	37	18	2
10	Parkinsonism	M	67	23	4
11	Anal fissure	M	64	15	1
12	Tendon suture	M	38	13	2
13	Renal glycosuria	M	34	11	1
14	Skin graft	M	52	22	2
15	Finger amputation	M	26	22	6
16	Control (student)	M	31	14	2
17	Control (student)	M	24	18	3
18	Tonsillitis	M	20	17	3
19	Control (technician)	F	23	14	4
20	Control (technician)	F	23	16	4

(all of whom had some evidence of hepatic disease) the forty-five minute sample was within Mateer's upper normal limits of 4 per cent retention, although the fifteen-minute sample revealed more than 25 per cent BSP retention.[2] Attention is called particularly to Patient 21 with congestive heart failure. In our experience, increased BSP retention in the presence of congestive heart failure is likely to be more evident in the fifteen-minute than in the forty-five minute sample. The decrease in BSP retention that occurs with recovery from congestive heart failure, demonstrated here, has been repeatedly observed.

TABLE III. BROMSULFALEIN RETENTION IN INDIVIDUALS HOSPITALIZED WITH MISCELLANEOUS DISORDERS

PATIENT	DIAGNOSIS	SEX	AGE (YR.)	BROMSULFALEIN RETENTION (%)		COMMENTS
				15 MIN.	45 MIN.	
1	Goiter	M	44	29	1	B.M.R., + 22%
2	Hydrocele	M	55	37	18	Wine, quart daily for 10 yr.
3	Hyperthyroidism	F	32	34	12	B.M.R., + 32%
4	Cirrhosis	M	70	52	15	Whiskey, quart daily for 4 mo.
5	Bile duct carcinoma	M	69	32	27	Icterus index 40 units
6	Cirrhosis	M	60	30	18	Serum protein, 5.5 Gm. %; ascites
7	Cirrhosis	M	60	36	4	Cephalin flocculation, 3+
8	Pernicious anemia	M	58	32	1	R.B.C., 1.4 million
9	Carcinoma of rectum	M	69	27	3	Hepatomegaly
10	Alcoholism, diabetes	M	66	41	8	No hepatomegaly
11	Finger amputation	M	60	32	8	Formerly drank quart wine daily
12	Trophic ulcers	M	16	30	8	Spina bifida
13	Bronchiectasis	M	39	27	7	Post-operative, lobectomy
14	Carcinomatosis	M	39	31	20	Primary site undermined
15	Hepatitis	F	39	77	49	Icterus index 30 units
16	Hepatitis	F	26	66	47	Icterus index 100 units
17	Hyperthyroidism	F	34	50	22	B.M.R., + 42%
18	Hyperthyroidism	F	44	42	12	B.M.R., + 28%
19	Gallstones	F	44	38	7	Asymptomatic
20	Cholecystitis	F	40	46	6	Subsiding; afebrile; no jaundice
21	Congestive heart failure	M	43	32	2	In severe failure, congestive hepatomegaly
				6	1	After recovery 6 wk. later

TABLE IV. CHANGES IN BSP RETENTION DURING HOSPITALIZATION FOR ALCOHOLISM

CASE	AGE	SEX	DURATION OF CHRONIC ALCOHOLISM	DURATION OF DRINKING BOUT IMMEDIATELY BEFORE HOSPITALIZATION	BROMSULFALEIN RETENTION ON ADMISSION IN PER CENT RETENTION		DURATION OF HOSPITALIZATION	BROMSULFALEIN RETENTION ON DISCHARGE IN PER CENT RETENTION		DIETOTHERAPY
					15 MIN.	45 MIN.		15 MIN.	45 MIN.	
1	46	F	10 yr.	3 mo.	42	4	2 wk.	20	0	House diet without supplements
2	48	F	16 yr.	4 wk.	45	10	2 wk.	30	0	High protein-CHO diet with supplements
3	39	M	2 yr.	2 wk.	40	4	2 wk.	20	3	High protein-CHO diet with supplements
4	45	M	4-5 yr.	2 wk.	34	10	10 days	21	7	High protein-CHO diet with supplements
5	48	M	20 yr.	6 wk.	34	10	3 wk.	18	4	House diet without supplements
6	53	M	25 yr.	3 wk.	38	4	2 wk.	18	2	High protein-CHO diet with supplements
7	50	M	20 yr.	18 days	32	2	1 wk.	10	0	House diet without supplements
8	77	M	4 yr.	1 yr.	80	10	4 wk.	32	6	High protein-CHO diet with supplements
9	46	M	20 yr.	4 wk.	38	4	2 wk.	18	5	High protein diet with supplements
10	45	F	8 yr.	4 wk.	60	30	2 wk.	27	15	High protein-CHO diet with supplements
11	48	M	20 yr.	6 wk.	72	40	2 wk.	60	18	High protein-CHO diet with supplements
12	53	F	14 yr.	8 mo.	95	80	5 wk.	54	30	High protein-CHO diet with supplements
13	57	M	20 yr.	2 wk.	36	12	3 wk.	26	4	House diet without supplements
14	50	M	18 yr.	6 mo.	97	95	6 wk.	40	22	High protein-CHO diet with supplements
15	53	F	12 yr.	4 mo.	42	16	2 wk.	18	4	House diet without supplements
16	70	M	20 yr.	3 mo.	52	14	1 wk.	28	12	High protein-CHO diet with supplements
17	62	M	18 yr.	3 mo.	54	16	2 wk.	28	8	House diet without supplements

Table IV records the BSP retention of seventeen chronic alcoholics hospitalized for acute intoxication. At the time of admission to the hospital the BSP retention in forty-five minutes was 4 per cent or under in five cases. However, the fifteen-minute sample revealed 30 per cent or more BSP retention in every instance.

With abstinence from alcohol, and with sedation as necessary (chloral hydrate, paraldehyde) and an adequate dietary regimen, there was a distinct decrease in BSP retention in every case. The decrease in BSP retention was particularly striking in the fifteen-minute sample in Cases 1, 3, 4, 6, 10, and 13 which had exhibited only slight increases in BSP retention before the inception of therapy.

Six of these individuals (Cases 1, 5, 7, 13, 15, 17) received only the routine hospital diet without a high protein diet or vitamin supplements. The eleven other subjects received daily a high protein diet (120 Gm. or more), 6 Gm. of

choline dihydrogen citrate in divided doses, and 6 capsules each containing 110 mg. ascorbic acid, "d" calcium pantothenate 9.5 mg., choline chloride 20.5 mg., folic acid 5.5 mg., inositol 54.5 mg., niacinamide 110 mg., pyridoxine HCl 1.0 mg., riboflavin 13.0 mg., thiamin HCl 16.5 mg., and liver powder 220.0 milligrams.*

In this experiment, improvement in liver function as evidenced by a decrease in BSP retention occurred both in those patients receiving the high protein diet with supplements and in those receiving the routine hospital diet without choline and vitamin supplements. Although the clinical impression obtained that the patients on the high protein, high vitamin regime responded more rapidly, no conclusive evidence in this regard was observed.

SUMMARY

These data indicate that sufficient additional information is obtained from the fifteen-minute serum sample in the BSP retention test to justify its use along with the forty-five minute sample.

It is suggested that with the BSP method used in this study 25 per cent retention in the fifteen-minute sample is probably the upper limit of normal. As noted by Mateer and associates,[2] 4 per cent retention marks the upper limit of normal in the forty-five minute sample.

The decrease in BSP retention following recovery from acute intoxication in a group of chronic alcoholics is reported. This decrease in BSP retention was not markedly different in patients receiving the routine hospital diet from those receiving a high protein diet with choline and vitamin supplements.

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*The choline dihydrogen citrate and the vitamin capsules were supplied through the courtesy of Dr. Stanton M. Hardy of Lederle Laboratories, Inc., New York, N. Y.

THE EFFECT OF ORAL ADMINISTRATION OF CASEIN HYDROLYSATE ON THE TOTAL CIRCULATING PLASMA PROTEINS OF MAN

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NUMEROUS investigators report that diseases and physical injury can effect not only a decrease in plasma protein concentration but also a change in the composition of the plasma proteins.[1-6] The outstanding changes common to many of these situations are a decrease in the per cent of albumin and an increase in the per cent of alpha globulins such as are commonly observed in protein depletion.[7-9] Consequently, the changes in the plasma protein patterns in many situations may be due to protein depletion rather than to an inherent specificity of a given disease. Should this be the case, in fact, then the feeding of a suitable protein should result in a return toward normal of the plasma protein pattern without the necessity of effecting at the same time any significant change in the disease itself. Some selection of the protein may be necessary since it would appear that different proteins may cause regeneration of different plasma proteins to different degrees.[10, 11]

The ability of a protein or a hydrolysate of a protein to stimulate the regeneration of various plasma proteins has some importance in itself, since hydrolysates have come into clinical use.[12-14] The present investigation is concerned with a study of the plasma protein pattern in a variety of clinical cases and with the ability of a casein hydrolysate* used as the sole source of protein to cause restoration of abnormal patterns to normal.

EXPERIMENTAL METHODS

Plasma volumes were determined by the method of Gregersen and Stewart[15] using Evans blue dye (T-1824). The total protein concentration of plasma was determined by the micro-Kjeldahl method with selenium and copper sulfate as catalysts. For electrophoretic analysis of the plasma proteins, about 10 ml. of plasma were diluted with an equal volume of diethylbarbiturate buffer at pH 8.4 and ionic strength of 0.1 and then dialyzed against 2.0 liters of the same buffer for twenty-four hours in a cold room. The scanning technique and the method of resolution of Longsworth[16] were used.

Chemical and Biologic Characteristics of the Hydrolysate Used.—The characteristics of the casein hydrolysate used in these studies were determined because a protein or protein hydrolysate would be expected to regenerate plasma protein only if it contains the essential amino acids in adequate proportions, as evidenced by chemical analysis and by ability to support nitrogen balance

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and to promote growth of young animals at a low total dosage. The essential amino acid content of the hydrolysate is given in Table I. The strepogenin content, the nitrogen balance index as observed in dogs, and the growth efficiency obtained in rats are given in Table II.

These data demonstrate that the hydrolysate used is equal to a high grade of edible casein in nutritive properties, casein itself being an adequate protein for most nutritive purposes. This view is also borne out by comparative feeding of casein and this hydrolysate to protein depleted rats*[17] and by its ability to support nitrogen balance in man at a level of 0.18 to 0.20 Gm. nitrogen per kilogram per day.[18]

TABLE I. ANALYSES OF THE TEN ESSENTIAL AMINO ACIDS AND CYSTINE AND TYROSINE IN A CASEIN HYDROLYSATE

AMINO ACIDS	PER CENT FOUND*	
	"AS IS" BASIS	H_2O AND ASH-FREE BASIS
Arginine†	2.9	3.2
Lysine†	6.7	7.3
Tryptophane‡	1.1	1.2
Threonine†	4.3	4.7
Histidine†	2.7	2.9
Phenylalanine†	4.6	5.0
Valine†	5.9	6.4
Methionine†	2.7	2.9
Isoleucine†	5.9	6.4
Leucine†	8.7	9.5
Cystine§	0.4	0.43
Tyrosine‖	2.8	3.0

The authors are indebted to Dr. R. D. Greene of E. R. Squibb & Sons for the determination of the amino acids.

*The total nitrogen content is equal to 13.5 per cent of which 25 to 30 per cent is in the form of free amino nitrogen.

†Stokes, J. L., Gunness, M., Dwyer, I. M., and Caswell, M.: J. Biol. Chem. **160**: 35, 1945.

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TABLE II. COMPARISON OF THE BIOLOGIC PROPERTIES OF CASEIN AND THE CASEIN HYDROLYSATE

TESTS	SUBSTANCE TESTED	
	CASEIN*	CASEIN HYDROLYSATE
Strepogenin (Units/Gm.)	4-5	4-5†
Nitrogen balance index	0.80	0.80‡
Growth efficiency	2.2	2.2§

*The casein used was a high grade of edible casein from the Borden Company, New York, N. Y.

†Woolley, D. W.: J. Biol. Chem. **162**: 383, 1946.

‡Allison, J. A.: J. Nutrition **29**: 413, 1945.

§We are indebted to Dr. A. Black of E. R. Squibb & Sons for making the growth efficiency tests.

RESULTS

The determination of the ability of casein hydrolysate to promote plasma protein regeneration and the estimation of the type of plasma protein regenerated were made by feeding experiments wherein the casein hydrolysate was administered to two groups of hypoproteinemic patients, a total of twenty-eight subjects. Dextrimaltose was given so that the daily energy intake of the patients was at least 3,000 calories. The plasma protein concentration and electrophoretic

*The authors are indebted to Dr. Paul R. Cannon and associates for the determinations.

analyses were performed on samples of plasma taken before and after two to four weeks of administration. The patients in Group I as a whole were hypoproteinemic* and not hypoalbuminemic, and the albumin-globulin ratios were essentially normal. Therefore, in order to study the effect of casein hydrolysate feeding, their total circulating plasma proteins were determined before and after the hydrolysate feeding period. The patients in Group II were not only hypoproteinemic but also markedly hypoalbuminemic. The albumin-globulin ratio of the plasma was considerably below normal in all patients. Hence the increase of albumin content following the administration of casein hydrolysate was marked. The other components of the plasma protein were also determined before and after the hydrolysate therapy.

Group I. The Increase of Total Circulating Proteins Following the Oral Administration of Casein Hydrolysate to Hypoproteinemic Patients.—Casein hydrolysate was fed to three patients after herniotomy at a low level of nitrogen intake (0.2 Gm. nitrogen per kilogram body weight per day) for about two weeks. (Table III.) These patients, though in nitrogen equilibrium, were unable to increase their circulating plasma protein. Two other patients after herniotomy were fed at a moderate level of nitrogen intake (0.5 and 0.6 Gm. nitrogen per kilogram body weight per day). A pronounced increase of plasma proteins was observed in both patients. Subsequent studies used a high dosage level ranging from 0.6 to 1.0 Gm. nitrogen per kilogram body weight per day for periods ranging from ten days to seven weeks.

Total circulating proteins of fifteen patients characterized by different types of disease were determined before and after hydrolysate feeding (Table IV). An increase of at least 15 per cent in plasma protein was obtained in ten of the fifteen patients. An increase of somewhat more than 10 per cent, the limit of accuracy of the estimation, was observed in the remaining five. This increase involved both albumin and globulin fractions in all subjects but one, Patient An. J. Hydrolysate feeding did not produce any consistent or significant changes in the per cent of any particular globulin fractions.

TABLE III. DETERMINATION OF TOTAL CIRCULATING PLASMA PROTEINS OF PATIENTS AT DIFFERENT LEVELS OF NITROGEN INTAKE

PATIENT	NITROGEN DOSAGE (GM./KG./DAY)	NITROGEN GAIN (GM./KG./DAY)	DAYS OF TREATMENT	PER CENT GAIN (+) OR LOSS (−) OF TOTAL CIRCULATING PROTEIN	A/G*
La.	0.2	0.0	12	−10	1.08
Ka.	0.2	−0.06	13	− 5	1.13
Ma.	0.2	−0.02	14	− 4	----
Si.	0.5	+0.13	18	+19	1.44
Be.	0.6	+0.2	7	+18	1.04

*Ratio of albumin to globulin before treatment, as determined by the immunologic method.

Group II. Changes of Plasma Patterns Following the Administration of Casein Hydrolysate to Hypoproteinemic and Hypoalbuminemic Patients.—Eleven patients with albumin ranging from 20 to 43 per cent of the total plasma protein were fed with hydrolysate at an intake of 0.6 to 1.0 Gm. nitrogen per

*The term hypoproteinemia is used here to mean abnormally low in total circulating proteins, which takes into account not only plasma protein concentration but also plasma volume.

TABLE IV. PLASMA PROTEIN REGENERATION OF PATIENTS FOLLOWING CASEIN HYDROLYSATE ADMINISTRATION

PATIENT	DAYS OF TREATMENT	PLASMA PROTEIN (GM./100 ML.)	PLASMA VOLUME (ML.)	TCP	TCA	TCG	DIAGNOSIS
St.	0	5.81	3592	209	100	109	Peptic ulcer
	10	6.58	3745	246	123	123	
		Change of plasma protein		+37	+23	+14	
Gl.	0	5.71	2965	169	93	76	Malnutrition
	15	6.24	3206	200	116	84	
		Change of plasma protein		+31	+23	+ 8	
An. G.	0	5.81	3410	198	139	59	Muscular dystrophy
	15	7.50	5500	413	256	157	
		Change of plasma protein		+215	+117	+98	
Si.	0	7.13	3555	253	149	104	Gastric ulcer
	17	6.60	4715	311	180	131	
		Change of plasma protein		+58	+31	+27	
Go.	0	6.63	2410	160	82	78	Gastric ulcer
	21	7.25	3050	221	106	115	
		Change of plasma protein		+61	+24	+37	
Mi.	0	5.19	2740	142	61	81	Gastric ulcer
	21	6.38	4340	277	122	155	
		Change of plasma protein		+135	+61	+74	
Da.	0	5.36	3050	163	70	93	Malnutrition
	25	7.01	3432	241	135	106	
		Change of plasma protein		+78	+65	+13	
Ha.	0	5.64	2873	162	73	89	Malnutrition; ulcer of leg
	37	7.37	3073	226	108	118	
		Change of plasma protein		+64	+35	+19	
Su.	0	6.02	3042	183	71	112	Malnutrition
	43	6.01	3840	231	99	132	
		Change of plasma protein		+48	+28	+20	
We.	0	5.46	2630	144	62	82	Gastritis
	52	6.25	2665	167	78	89	
		Change of plasma protein		+23	+16	+ 7	
He.	0	7.13	2960	211	116	95	Cap ulcer
	15	7.25	3168	230	127	103	
		Change of plasma protein		+19	+11	+ 8	
An. J.	0	6.81	3200	218	144	74	Muscular dystrophy
	21	7.38	3325	245	127	118	
		Change of plasma protein		+27	-17	+44	
Ro.	0	7.56	3250	246	118	128	Duodenal ulcer
	21	7.81	3520	275	127	148	
		Change of plasma protein		+29	+ 9	+20	
Harr.	0	6.29	2829	178	112	66	Malnutrition
	22	6.02	3261	196	122	74	
		Change of plasma protein		+18	+10	+ 8	
Ga.	0	7.64	2138	164	110	54	Gastric ulcer
	30	7.00	2570	180	121	59	
		Change of plasma protein		+16	+11	+ 5	

TCP, Total circulating protein; TCA, total circulating albumin; TCG, total circulating globulin.

TABLE V. EFFECT OF ORAL FEEDING OF CASEIN HYDROLYSATE ON PLASMA PROTEIN OF HYPOALBUMINEMIC PATIENTS

PATIENT	DAYS OF TREATMENT	PLASMA PROTEIN (GM./100 ML.)	PLASMA VOLUME (ML.)	ALBUMIN/GLOBULIN	PLASMA PROTEIN COMPONENTS (GM. %)						DIAGNOSIS
					ALBUMIN	$ALPHA_1$ GLOBULIN	$ALPHA_2$ GLOBULIN	BETA GLOBULIN	FIBRINOGEN	GAMMA GLOBULIN	
Eg.†	0	7.40	----	0.35	1.92	0.59	1.11	0.52	0.44	2.81	Pemphigus
	15	6.75	----	0.49	2.23	0.68	0.95	0.34	0.47	2.09	
	43	7.68	----	0.67	3.07	0.46	1.00	0.69	0.38	2.07	
Yo.*	0	4.62	----	0.25	0.92	0.60	1.43	0.55	0.55	0.55	Cancer
	17	6.52	----	0.59	2.41	0.59	0.85	1.04	0.65	0.98	
	20	7.00	----	0.67	2.80	0.56	0.77	1.19	0.77	0.91	
Da.*	0	5.36	3050	0.75	2.30	0.54	0.80	0.64	0.21	0.86	Malnutrition
	25	7.01	3432	1.27	3.93	0.35	0.84	0.49	0.28	1.12	
Lo.†	0	6.76	2640	0.49	2.23	0.47	1.28	1.15	0.27	1.35	Pulmonary tuberculosis
	31	6.72	2498	1.13	3.56	0.34	0.20	0.67	0.67	1.28	
	68	6.40	2462	1.08	3.33	0.45	0.38	0.58	0.64	1.02	
Su.*	0	6.02	3042	0.64	2.35	0.54	0.90	0.60	0.42	1.20	Malnutrition
	43	6.01	3840	0.75	2.58	0.60	1.14	0.84	0.06	0.78	
Po.*	0	5.89	----	0.47	1.88	0.53	1.06	0.71	0.24	1.47	Diabetes mellitus
	60	8.00	----	0.82	3.60	0.32	1.04	0.72	0.32	2.00	

*The casein hydrolysate used was Casein Hydrolysate (Squibb.)
†The casein hydrolysate used was Amigen, Mead Johnson & Company, Evansville, Ind.

TABLE VI. FAILURE OF CASEIN HYDROLYSATE THERAPY TO PROMOTE THE REGENERATION OF PLASMA PROTEINS OF PATIENTS AT THE TERMINAL STAGES OF DISEASE

PATIENT	DAYS OF TREATMENT	PLASMA PROTEIN (GM./100	PLASMA VOLUME (Ml.)	TCP (GM.)	TCA (GM.)	TCG (GM.)	ALBUMIN/ GLOBULIN	PLASMA PROTEIN COMPONENTS (GM. %)						DIAGNOSIS
								ALBUMIN	$ALPHA_1$ GLOBULIN	$ALPHA_2$ GLOBULIN	BETA GLOBULIN	FIBRINOGEN	GAMMA GLOBULIN	
Jo.	0	7.44	3562	265	74	191	0.39	2.08	0.97	1.19	0.89	0.45	1.86	Cancer
	12	7.32	3538	259	62	197	0.32	1.76	0.88	1.24	1.17	0.44	1.83	
Hi.	0	7.32	4683	343	93	250	0.37	1.98	0.73	1.46	1.10	0.51	1.54	Tuberculosis
	13	6.70	5053	339	85	254	0.33	1.68	0.74	1.41	0.80	0.34	1.74	
Sa.	0	4.90	2525	124	33	91	0.37	1.32	0.78	0.98	0.39	0.39	1.03	Terminal ileitis
	13	4.69	2611	122	29	93	0.32	1.13	0.84	0.70	0.52	0.66	0.84	
	19	5.33	2166	115	32	83	0.39	1.49	0.96	0.75	0.59	0.64	0.91	
Gr.	0	5.39	3956	213	47	166	0.28	1.19	0.92	1.13	0.59	0.59	0.97	Transverse myelitis
	2	5.68	4080	232	58	174	0.33	1.42	0.91	1.25	0.62	0.62	1.02	
	8	5.31	3822	203	45	158	0.28	1.17	0.91	1.17	0.58	0.53	0.96	
	24	5.16	3918	202	42	160	0.27	1.08	0.72	0.98	0.62	0.72	1.03	

TCP, Total circulating protein; TCA, total circulating albumin; TCG, total circulating globulin.

kilogram body weight per day. The patients in this series were divided into two groups. Subgroup A, six subjects, responded to the hydrolysate therapy, and subgroup B, five subjects, failed to respond to the casein hydrolysate feeding; the latter were all in the terminal stages of their diseases.

Subgroup A: The results given in Table V demonstrate that administration of this hydrolysate brought about a rapid increase in plasma protein concentration or in plasma volume in all patients but one (Patient Lo.). The plasma volume of Patient Eg. was not determined; therefore its change, if any, is not known. The albumin content in terms of gram per cent was increased in all cases. As would be expected, the increase was most marked where hypoalbuminemia and hypoproteinemia were most severe, that is, where albumin-globulin ratio and plasma protein concentration were the lowest.

The response of Patient Lo. to the hydrolysate therapy is of particular interest because there was neither increase in protein concentration nor in plasma volume after sixty-eight days of the hydrolysate therapy. Nevertheless, there was a very significant increase in albumin-globulin ratio. The increase in albumin, therefore, was made at the expense of the globulins.

Subgroup B: Four hypoalbuminemic patients with albumin content of 30 per cent or less were fed with casein hydrolysates at a level of as much as 0.6 Gm. nitrogen per kilogram body weight per day for about two weeks or longer. During this period the patients were in positive nitrogen balance and retained as much as 0.4 Gm. nitrogen per kilogram body weight per day. However, in spite of the large nitrogen retention, the pattern of the plasma proteins of these patients failed to approach normal. (Table VI.) The albumin-globulin ratio remained low and there was no significant change in the total circulating albumin or globulins. No one of these four patients who did not respond to the therapy survived for any great length of time following these observations. The results may indicate that during the advanced stages of certain diseases patients may be unable to utilize an otherwise adequate mixture of amino acids and polypeptides for the synthesis of plasma proteins.

DISCUSSION

The patients used in this study reacted much the same as do experimental animals to gradual protein depletion. Consequently, results are consistent with the belief that of the systemic manifestations of diverse diseases the abnormalities observed in the pattern of the plasma proteins and in their amount are in part the results of a general protein deficiency.

A casein hydrolysate which contains all the essential amino acids and high biologic values as determined in laboratory animals and in man was administered to patients to correct the abnormal plasma patterns. The patients used constituted a rather heterogeneous group. They were characterized by different degrees of hypoproteinemia and by different types of disease; in addition, the period of hydrolysate therapy was not always uniform. However, in spite of these variations the results are sufficiently striking to indicate that, in general, the administration of a suitable casein hydrolysate at a sufficiently high level to hypoalbuminemic patients results initially in a rapid increase in plasma

albumin content. Such a result is not to be expected in the terminal phases of certain diseases. When the hypoalbuminemia is not severe, the feeding of casein hydrolysate produces an increase of both plasma albumin and of plasma globulin. This effect on the globulin fractions may have special significance because of the possible relationship of certain globulins and certain of the so-called vital functions of the body related to the synthesis of certain of the hormones, the enzymes and the antibodies.

The failure of some patients to regenerate either albumin or globulins following casein hydrolysate therapy in spite of positive nitrogen balance is of interest. This fact is in line with other information which indicates that plasma proteins are not as readily synthesized as some other tissue proteins.

SUMMARY

Casein hydrolysate has been given to twenty-eight hypoproteinemic and hypoalbuminemic patients with a variety of diseases. It promotes the regeneration of both the plasma albumin and globulin fractions. In six cases of severe hypoalbuminemia, the albumin deficiency was corrected rapidly. Five hypoalbuminemic patients at the terminal stages of their diseases were able to utilize the hydrolysate to maintain a positive nitrogen balance but not to regenerate plasma proteins.

These results are consistent with the belief that diverse medical and surgical conditions are characterized by varying degrees of protein depletion which can be corrected by large amounts of casein hydrolysate.

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STUDIES ON THE MINIMUM PROTEIN REQUIREMENTS OF ADULT DOGS

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THE protein requirements of adult animals including man have been investigated almost exclusively by nitrogen balance studies. This is a reasonable method since only the losses through the skin and those involved in the growth of hair and skin are not taken into account. These are undoubtedly small. However, little work has been directed toward the determination of the proper conditions under which the studies should be made. It is known that when an animal is fed a low protein diet, the urinary nitrogen falls rapidly during the first few days and continues to fall for a considerable period, but at a decreasing rate. It seems obvious that the amount of protein required to balance these losses will depend upon the time the studies are made, or the degree of nitrogen depletion of the subject.

The fundamental studies of Terroine and co-workers,[1-3] Sorg-Matter,[2] Ashworth and Brody,[4] and Smuts[5] have shown that eventually the nitrogen excretion reaches a minimum which is closely correlated with the basal metabolism of the subject. This minimum may be considered the endogenous level of nitrogen metabolism, and corresponds to 1.4 to 2.0 mg. of nitrogen per basal calorie. Since this appears to be minimum nitrogen excretion, it is reasonably certain that the minimum nitrogen requirement cannot be below these levels. For an individual with a basal metabolism of 1,500 calories per day, the minimum daily protein requirement would thus be between 13 and 19 Gm., assuming complete utilization. Most proteins are of course not completely utilized and the minimum requirements are above these levels. The amount above may be calculated from a consideration of the degree of digestibility and the biologic value of the protein fed. The recent studies of Bricker, Mitchell, and Kinsman[6] and Hegsted, Tsongas, Abbott, and Stare[7] indicate that the protein requirement of human adults is in this range, that is, endogenous requirement plus corrections for digestibility and biologic value, and have yielded minimum values considerably below the previously accepted figures.

However, it must be recognized that although nitrogen balance is an apparently adequate criterion of whether an animal is being maintained with regard to nitrogen, it tells nothing of the condition of the animal thus being maintained. It is not impossible that continued maintenance of an animal in a depleted state may lead to serious consequences. From the studies of Addis and associates[8-10] it would appear that animals depleted to endogenous levels of nitrogen excretion would have severely depleted livers, and Elman and co-workers[11] have shown that plasma protein levels begin to fall soon after low

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nitrogen diets are started. It is important to know how serious these consequences are and whether the results obtained upon such depleted animals are interpretable in terms of normal animals since Bricker and co-workers[6] depleted two of their human subjects to apparently endogenous levels.

This paper represents the first of a series of studies designed to investigate more completely the response of animals to low protein diets. In addition to nitrogen excretion we have studied changes in blood proteins, fluid volumes, and liver pathology at the conclusion of the experiments. The dogs used were partially depleted of nitrogen, as evidenced by urinary nitrogen excretion and plasma protein levels, and then fed whole egg protein at a level which supplied approximately 2 mg. of nitrogen per basal calorie. It is concluded that although this level of nitrogen eventually allowed nitrogen balance, the degree of nitrogen depletion achieved during or prior to the study was sufficiently severe that the results may not be applicable to normal animals. Similar studies done without nitrogen depletion will be reported subsequently.

EXPERIMENTAL

General Plan.—Two male and four female adult dogs weighing between 7 and 14 kilograms were used for this study. They were housed in metabolic cages and were weighed every other day. The daily amount of synthetic diet given allowed approximately 80 calories per kilogram of body weight per dog per day. When protein was fed, an isocaloric amount of basal diet was replaced by the protein. The purified basal diet was composed of sucrose, 86 per cent; corn oil (Mazola), 8 per cent; salt mixture, 4 per cent[12]; cellulose, 2 per cent; cod-liver oil, 2 per cent. Vitamins were added per 100 Gm. of ration as follows: thiamine hydrochloride, 0.4 mg.; riboflavin, 0.8 mg.; niacin, 3.0 mg.; pyridoxine hydrochloride, 0.4 mg.; calcium pantothenate, 1.5 mg.; choline chloride, 100.0 mg.; vitamin K, 0.01 mg.; biotin, 0.01 mg.; and folic acid, 0.025 mg. The actual amount of food consumed was recorded daily since there were periods when some dogs failed to eat the full allotment.

Before the experimental period was started, a high protein diet (16 per cent protein), prepared by replacing 44 per cent of sucrose in the basal diet with skimmed milk powder, was fed for two or three weeks to insure an optimum nutritional state. The nitrogen-free diet was then given for two or three weeks until the total protein content of plasma dropped slightly (about 1 per cent) below the initial level. This was supposed to be enough for depletion of the stored protein in tissues and body fluids, but was not considered severe protein deficiency. Spray-dried whole egg powder was then given to the dogs in amounts estimated to supply 2 mg. of nitrogen per basal calorie. The actual quantity for each dog was determined from Table 13.7 in Brody's text.[13] Basal metabolic measurements were not made. The total nitrogen intake varied from 68.3 to 83.6 mg. per kilogram of body weight. After thirty to forty days on this regime, the amount and source of nitrogen were changed as indicated in Figs. 1 to 6 and Table I. At the end of the study, all dogs were sacrificed by intravenous injection of air five to seven hours after feeding. Specimens of the livers were collected for biochemical and histologic studies.

At ten-day intervals during the entire study, plasma volume, and the total amount of circulating plasma protein, albumin, and hemoglobin were determined. Available fluid volumes (thiocyanate space) were obtained later in the study. Urine and feces were collected and pooled samples for each ten-day period analyzed for nitrogen.

Control data for the liver analyses and pathologic studies were obtained from five apparently normal dogs. Two of these had been fed a high protein diet previously mentioned for thirty days before autopsy. The other three were killed for other studies independent of nutritional studies. They had received a diet of dog chow and horse meat for unknown periods of time.

TABLE I. RESULTS OF THE ANALYSES OF THE

		DIETARY SCHEDULE				BODY WEIGHT (KG.)	FRESH LIVER WEIGHT	
		DIET	DAYS	DAILY NITROGEN INTAKE (MG./KG.)	DAILY NITROGEN INTAKE (AVERAGE MG./KG.)		(GM.)	(% BODY WEIGHT)
Normal dogs	A	High protein				14.5	503	3.47
	B	High protein				19.3	506	2.62
	C	Chow and meat				18.0	500	2.78
	D	Chow and meat				19.0	600	3.16
	E	Chow and meat				27.0	550	2.04
Experimental dogs	2	Nitrogen free	22	0				
		Egg protein	30	69.2				
		Egg protein + 0.25 Gm. methionine	15	70.0	67	14.0	535	3.97
		Egg protein	7	55.1				
	3	Nitrogen free	22	0				
		Egg protein	30	69.4	73	14.2	423	2.93
		Egg protein	20	78.6				
	4	Nitrogen free	22	0				
		Egg protein	30	68.3	101	14.8	446	3.01
		Casein	20	145.8				
	6	Nitrogen free	14	0				
		Egg protein	30	76.8	72	11.0	396	3.60
		Egg protein + 0.25 Gm. methionine	10	75.1				
		Egg protein	10	58.6				
	7	Nitrogen free	14	0				
		Egg protein	30	83.6	88	7.6	3.54	4.65
		Egg protein	20	91.7				

Methods.—The macro-Kjeldahl method was used for all nitrogen determinations. Hemoglobin was determined as acid hematin and plasma albumin by the Kjeldahl method after removal of globulin by 22 per cent sodium sulfate precipitation. Total plasma proteins were determined by the copper sulfate method of Philips and co-workers.[14] Hematocrit values were calculated from the hemoglobin value by multiplying by a factor of 2.95.[15]

For the determination of plasma volume the dogs were fasted twelve to sixteen hours to avoid lipemia, and 1 mg. of Evans blue dye per kilogram body weight was injected intravenously from a calibrated syringe. Blood samples were taken from another leg vein after ten minutes and mixed with heparin. Three tenths cubic centimeter of the plasma obtained after centrifugation were mixed with 5.7 c.c. of saline and read in the spectrophotometer at 620 millimicrons. Standard tubes containing known amounts of the dye were prepared simultaneously with plasma obtained before the dye was injected. When available fluid volumes were determined, sodium thiocyanate solution was injected through the same needle used for the Evans blue injection. Twenty milligrams of sodium thiocyanate per kilogram of body weight were given. Thiocyanate was determined by the method of Crandall and Anderson[16] in the plasma obtained forty-five minutes after injection.

Total blood volumes were calculated by the formula:

$$\text{Total blood volume} = \frac{\text{Plasma volume}}{100 - \text{venous hematocrit}}.$$

Total circulating hemoglobin, plasma protein or albumin were readily calculated by multiplying the concentration by the appropriate volume.

For the determination of liver lipids, the liver sample was ground with anhydrous sodium sulfate and extracted in a Soxhlet extractor for twenty-four to thirty hours with

LIVERS FROM NORMAL AND EXPERIMENTAL DOGS

LIVER NITROGEN							
TOTAL (GM.)	% FRESH WT.	% DRY WT.	MG./KG. BODY WT.	MG./GM. NONLIPID-NONGLYCOGEN LIVER	LIVER LIPIDS (% DRY WT.)	CHOLINE (MG./100 GM. DRY WT.)	GLYCOGEN (% DRY WT.)
20.552	4.090	13.11	1.422	–	8.3	–	–
17.003	3.363	14.69	0.880	–	17.6	–	–
16.348	3.270	11.55	0.910	–	–	–	–
17.844	2.974	10.05	0.940	–	–	–	–
19.036	3.461	11.69	0.710	–	–	–	–
9.389	1.757	5.39	0.670	102.6	8.9	602.3	38.6
8.692	2.080	6.74	0.630	89.6	12.3	764.2	13.4
9.527	2.138	6.75	0.670	132.6	5.1	266.5	43.9
8.849	2.234	7.20	0.83	138.9	13.7	511.2	34.4
6.263	1.768	5.70	0.87	113.0	10.0	–	31.0

chloroform. The chloroform extractable material was dried and weighed. The choline content was determined from another sample after grinding with anhydrous sodium sulfate and extraction with methanol by a modification of the reineckate method.[18]

The liver samples for glycogen determination were immediately sliced and dropped into a Pyrex tube of known weight containing a known amount of 30 per cent potassium hydroxide. The glycogen content was determined by the method of Good, Kramer, and Somogyi.[19]

Thin slices (3 to 5 mm.) of all livers were taken at random from any lobe and fixed in formaldehyde, formol-alcohol-picric acid solution of Rossman, and Zenker-acetic fixative. Sections were prepared by the paraffin or celloidin methods and stained with hematoxylin and eosin, eosin methylene blue, and by the Best carmine method for glycogen. Frozen sections were stained with Sudan IV.

RESULTS

The combined data on nitrogen balance, body weight, nitrogen and calories consumed, blood constituents, and body fluids are presented in Figs. 1 to 6. All dogs received egg protein at comparable levels for thirty days after the depletion period. At this time the changes in dietary regime indicated in the illustrations and in Table I were made. The results on egg protein will be discussed first.

Nitrogen Balance and Body Weight.—Nitrogen excretion studies were begun after the dogs had received the nitrogen-free diet for ten days. At this time the urinary nitrogen was clearly not at minimum levels since, with the exception of Dog 6, all the animals showed a stepwise reduction in nitrogen excretion in

subsequent periods. It is of interest that they showed this drop in nitrogen excretion even though egg protein was added to the diet. This result is in agreement with those of Miller,[20] Brush and co-workers,[21] and Allison and associates.[22] As has been discussed by these authors, this gives to egg protein a biologic value above 100. It is presumed that the animals would have reached this low level of nitrogen excretion eventually if continued on the nitrogen-free diet. Most of the dogs went into a slight positive balance eventually. Meanwhile the body weight increased slightly. It was the main purpose of the experiment to study the physiologic changes during this period.

Plasma Volume and Plasma Proteins.—Prior to depletion, the figures obtained for plasma volume, 41.2 to 60.7 c.c. per kilogram, total plasma proteins, 6.3 to 8.28 per cent, and total circulating plasma protein, 2.6 to 4.0 Gm. per

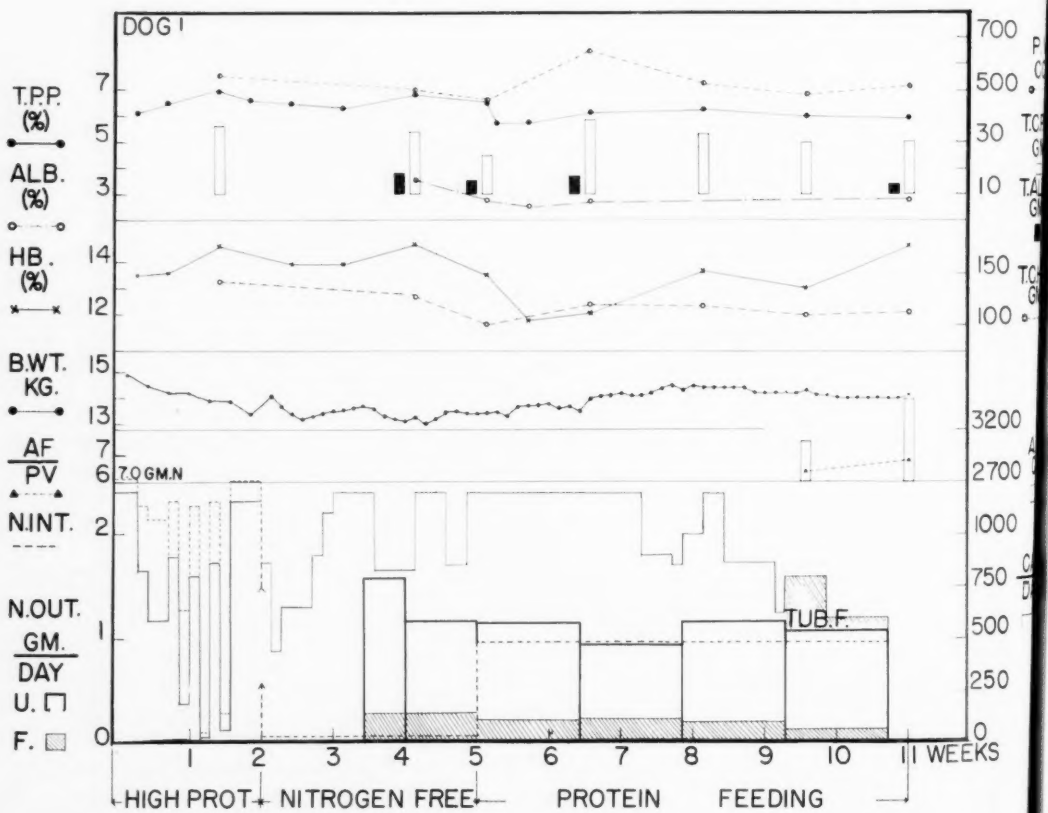

Fig. 1.—This figure summarizes the data obtained on Dog 1. T.P.P.%, Percentage of total protein in plasma; ALB.%, percentage of albumin in plasma; HB.%, per cent hemoglobin; B.W.T., body weight in kilograms; $\frac{AF}{PV}$, ratio of available fluid to plasma volume; N. INT., nitrogen intake, grams per day; N. OUT., total nitrogen output, grams per day; U., urinary nitrogen; and F, fecal nitrogen; P.V. c.c., plasma volume in cubic centimeters; T.C.P.P. Gm., total circulating plasma protein in grams; T.ALB. Gm., total circulating albumin in grams; T.C.H.B. Gm., total circulating hemoglobin in grams; A.F. c.c., available fluid volume in cubic centimeters; $\frac{CAL.}{DAY}$, calorie intake per day.

During the ninth week, tube feeding (TUB.F.) was resorted to to maintain nitrogen intake and partial caloric intake.

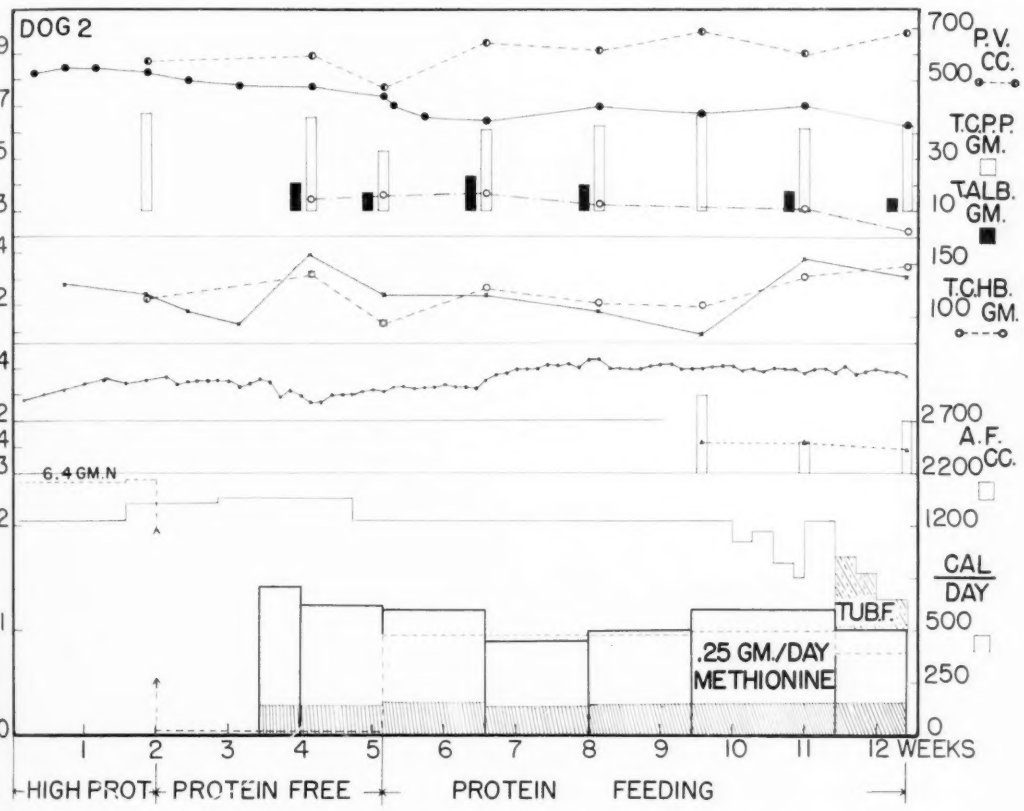

Fig. 2.—Data obtained on Dog 2. See Fig. 1 for interpretation.

kilogram are normal when compared with those reported in the literature.[17, 23-27] During the depletion period all of these decreased somewhat. This is of considerable interest since it indicates that there is a fall in plasma proteins before the minimum nitrogen excretion is reached. When egg protein was added to the diet, all of the dogs with the possible exception of Dog 4 showed a favorable response in total plasma protein. In most dogs this was shown by an increase in plasma volume while the percentage of protein remained essentially constant. In Dogs 4 and 7 the concentration increased somewhat with less marked or no change in plasma volume. The significance of the two types of response is not clear.

During the rest of the periods the level of protein was apparently sufficient to maintain both plasma volume and protein concentration. There is no evidence of a sustained fall indicating further protein depletion. Raising the level of nitrogen intake of Dogs 3 and 7 by 20 per cent may have caused a slight response, although in Dog 3 the control level of total plasma protein had been reached before the change was made. The last observation on this dog is probably complicated by the failure to maintain a maximum calorie intake. The same is certainly true for Dog 1.

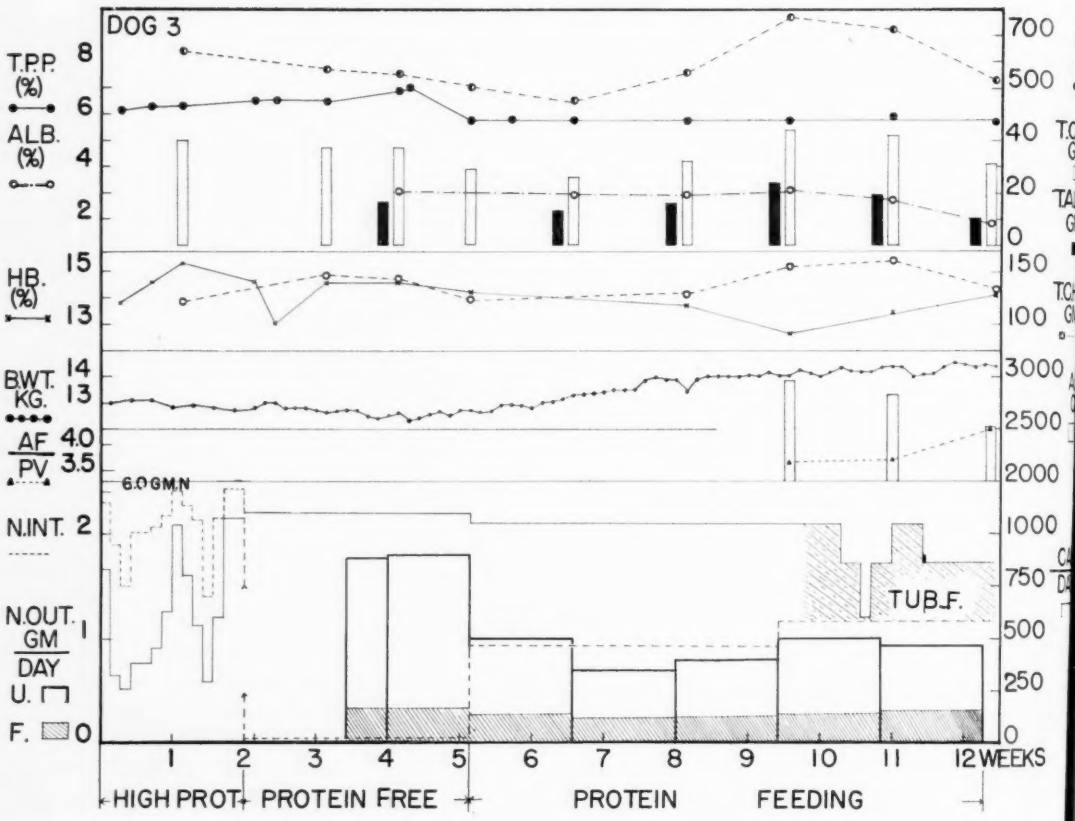

Fig. 3.—Data obtained on Dog 3. See Fig. 1 for interpretation.

Hemoglobin.—Almost all of the dogs showed some loss of hemoglobin during the depletion period, a total of about 10 Gm. per dog. With the exception of Dog 6, which was in relatively strong negative nitrogen balance throughout the study, there is little or no evidence of a progressively developing anemia after egg protein was fed. The percentage concentration of hemoglobin showed marked changes, especially with the changes in diet. The total circulating hemoglobin, in spite of the fact that it is a calculated figure, appears to be much more useful in studies of this type in evaluating the nutritional status. The fact that the hemoglobin concentration changes were chiefly the result of the changes in blood volume is apparent from the figures, and the constancy of the total circulating hemoglobin lends considerable support to the correctness, at least relatively, of our figures for plasma volume.

Available Fluid Volume.—Unfortunately, available fluid volumes were not determined early in the study, and some of the last values obtained may have been complicated by low calorie intakes. However, after the depletion period and thirty days on the low nitrogen regime, the available fluid volume varied from 198 to 237 and the plasma volume from 33.5 to 55.4 c.c. per kilogram of body weight. The ratio of available fluid to plasma volume varied from 3.8 to

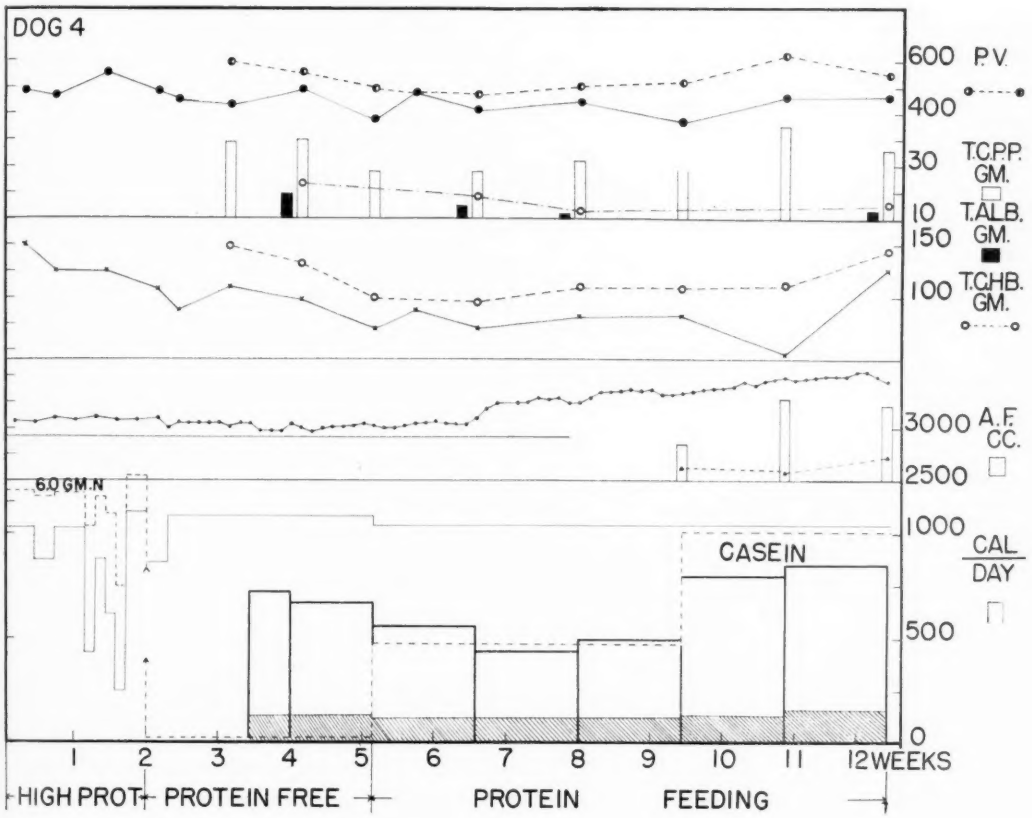

Fig. 4.—Data obtained on Dog 4. See Fig. 1 for interpretation.

6.8. It is of interest that Dogs 1 and 6 which were in the strongest negative nitrogen balance showed the highest ratios, 6.4 to 6.8. The values for the other dogs were in the normal range.[23, 26, 28, 29] The significance of later values for available fluid is problematical because of the failure of appetite in three dogs. This left only one dog receiving egg protein (at 20 per cent higher level than at the beginning). There appeared to be a steady increase in available fluid volume and ratio, although plasma volume was constant. The dog was in slight positive balance after a considerable period of negative balance. Nevertheless, this probably represented a continuing tendency toward edema. The opposite trend in Dog 6 was seen when nitrogen balance improved.

Methionine Supplementation.—Methionine was added to the diet of two dogs because of reports that this amino acid is effective in conserving body protein.[20-22] Dog 6 responded as expected. The urinary nitrogen fell markedly so that the dog was in balance and this was maintained in the following period even though the level of egg protein was decreased below that used during most of the study. A response in the circulating proteins was accompanied by an increase in plasma volume. Both the total hemoglobin and the hemoglobin content increased. The ratio of available fluid to plasma volume fell. In Dog 2

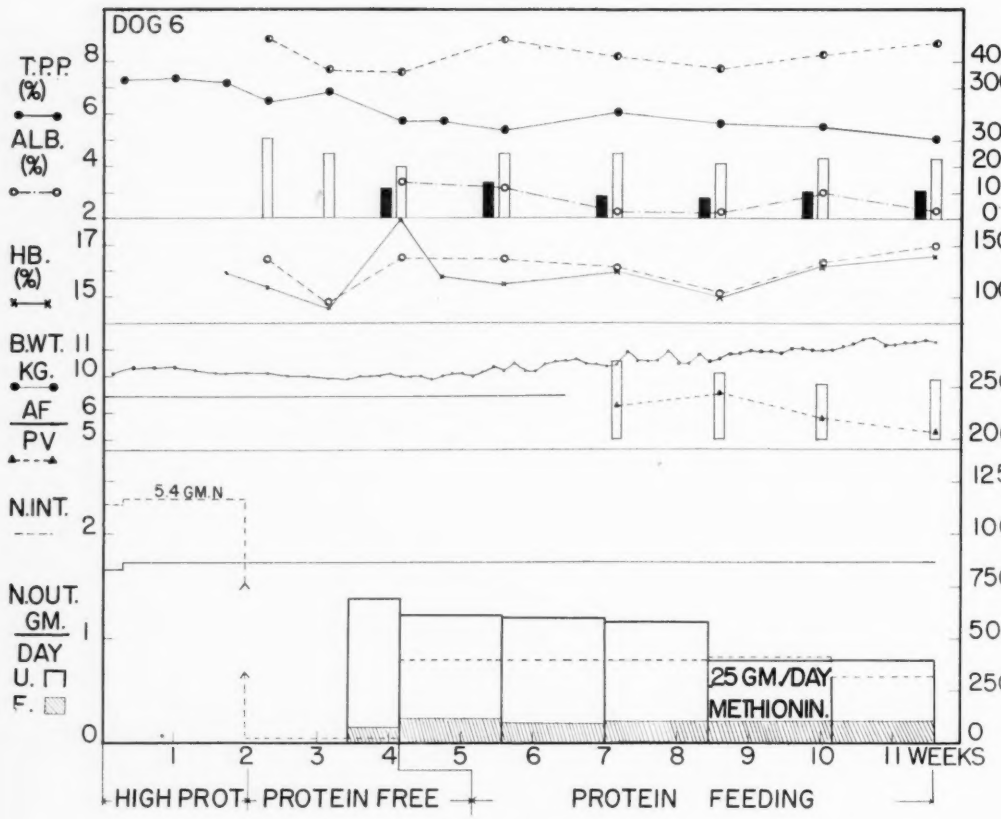

Fig. 5.—Data obtained on Dog 6. See Fig. 1 for interpretation.

the nitrogen balance became more negative. If the changes in plasma proteins and plasma volume are significant, they are opposite to those of Dog 6. The ratio of available fluid to plasma volume was maintained by a slight drop in available fluid. It should be noted that the ratio in Dog 2 was much lower than in Dog 6. There was, however, as in Dog 2, a favorable response in hemoglobin level and total hemoglobin.

It has been reported that the response to methionine is less marked after severe nitrogen depletion.[20] The data on nitrogen balance and total circulating protein indicate that Dog 6 was probably the more severely depleted. Thus the better response in Dog 6 was contrary to expectation. However, Dog 2 had previously shown a drop in urinary nitrogen to the endogenous level when given egg protein, while Dog 6 continued in strong negative balance. This may explain the difference in the two dogs at the time methionine was given, but the reason for the failure of Dog 6 to respond previously to egg protein remains unexplained.

Casein Supplementation.—Dog 4 was given a relatively high level of casein, supplying 2 Gm. of nitrogen per day. This was sufficient to throw the dog in strong positive balance. Coincident with this response, all of the values for

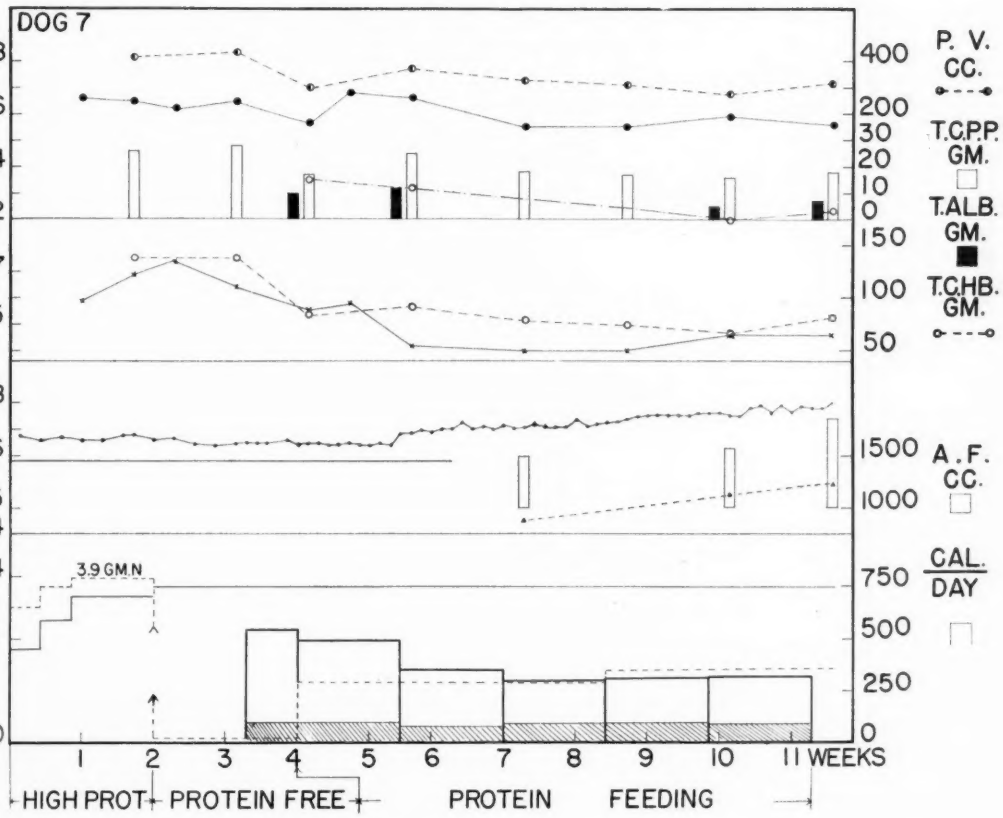

Fig. 6.—Data obtained on Dog 7. See Fig. 1 for interpretation.

plasma proteins, plasma volume, hemoglobin, and available fluid increased. The ratio AF/PV remained constant between 5 and 6. It may be worth while to call attention to the changes in total circulating protein and plasma volume, since this type of response was also observed in some of the dogs when they received egg protein previously. There appeared to be an increase in total protein as the primary response, and the changes in plasma volume followed this. In the next period the plasma protein apparently redistributed itself to body tissues. The total protein fell somewhat, together with the plasma volume, but not in proportion since the concentration of protein remained slightly higher than previously.

Liver Analyses.—The results for moisture, fat, glycogen, and choline are shown in Table I. The results obtained on the five supposedly normal dogs including two which had been given the purified diet containing 30 per cent skim milk powder for thirty days before they were killed are also presented. All animals had livers of normal fat and water content.[30, 31] The glycogen and choline varied greatly. The loss of protein of liver has been recognized as an early sign of fasting or protein deficiency.[8-10] Kosterlitz and Campbell[32, 33] found that the losses in protein, phospholipin, and nucleic acid during fasting and in protein deficiency represent a loss of liver cytoplasm. They found that

this loss of liver cytoplasm could be expressed by a curve containing an exponential and linear component. The linear decrease is thought to be a measure of endogenous metabolism, while the exponential decrease is probably due to loss of labile liver cytoplasm. These data suggest that the determination of liver nitrogen may be a simple and accurate index in experimental animals of the nutritional status with regard to protein. In Table I the nitrogen content of the livers has been expressed in several ways. If the nitrogen per gram of nonglycogen-nonfat liver solids is plotted against the average nitrogen intake of the

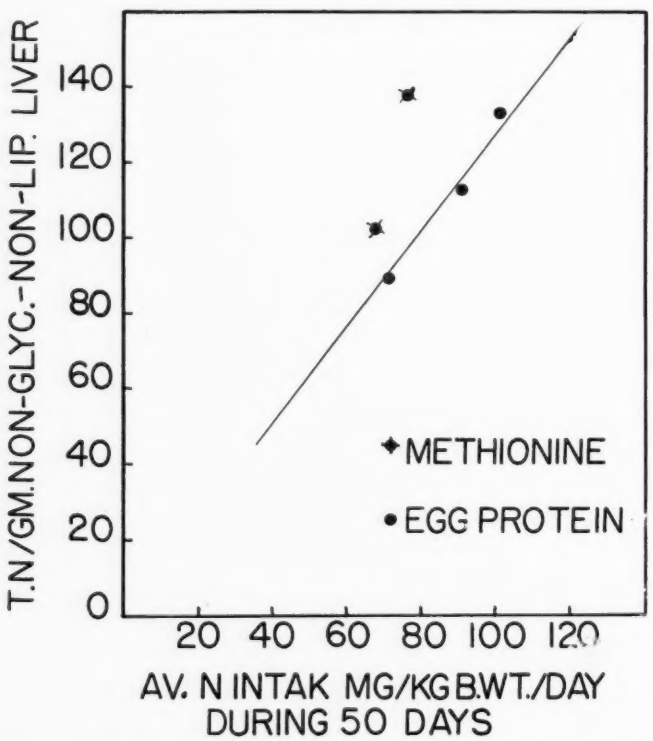

Fig. 7.—Diagram showing the relation between the average nitrogen intake, milligrams per kilogram of body weight per day, during the experimental period and the total liver nitrogen expressed as milligrams of nitrogen per gram of nonglycogen nonlipid liver.

dog during the entire study (including the supplements at the end of the experiment), there appears to be practically a straight-line relationship for three of the animals (Fig. 7). The two which fall above the line are those which received methionine supplements. These results agree with those recently reported by Brush and co-workers[21] and indicate a shift of protein toward the liver. It is interesting that this happened in Dog 2 although there was no apparent improvement in nitrogen balance.

The liver nitrogen contents per kilogram of body weight of our dogs are compared with various values taken from the literature in Table II. It is apparent that on this basis our animals are considerably below well-fed animals but much above severely depleted animals.

TABLE II. EFFECT OF DIET UPON THE NITROGEN CONTENT OF DOG LIVERS

SOURCE	DOG NO.	DIETARY CONDITIONS	AVERAGE LIVER NITROGEN (GM./KG. BODY WEIGHT)
Grund[34]	6	1.33-3.66 Gm. N per kg. body weight per day for 20 or 35 days	1.06
	4	Fasting for 13-19 days	0.57
Pugliese[35]	2	Normal	0.91
	2	Fasting for 20-24 days	0.60
	2	250-300 c.c. of milk given daily for 4 days after 28-30 days' fast	0.81
Authors	4	2 mg. N per basal calorie from egg protein for 40 or 50 days, following 2-3 weeks on nitrogen-free diet	0.72
	2	Same as Dog 4, plus methionine for 10 days	0.75
	2	Normal; high protein diet for 30-40 days	1.15
	3	Normal; dog chow and horse meat	0.89

Pathology.—

Gross: Except for a dark blue-green color imparted by the Evans blue dye to the liver, no striking changes were noted. All livers were smooth and the capsules glistened. The borders were sharp. Although all of the livers were firm, there was no increase in resistance to section. The weights of all livers are recorded and compared with body weight in Table I. The other organs showed no significant changes. The livers of the five normal dogs also showed no significant change.

Microscopic: The changes found in the livers of experimental animals 2, 3, 4, 6, and 7 differed only quantitatively, if at all, and were similar to those described by Elman and Heifitz[30] in dogs on protein deficient diets.

There was marked swelling of liver cells and the cell membrane was well defined and intensely stained. So extensive was the swelling of liver cells that the sinusoids were largely obliterated. Most striking of all changes was the marked rarefaction of cytoplasm which was represented by a few eosinophilic granules of the cytoplasm identified as ribonucleic acid by various techniques (see review by Greenstein[36]).

The liver cell nuclei were uniformly small, central, and round or oval with a scant amount of fine, evenly dispersed chromatin material and prominent, often multiple, nucleoli. In many instances binucleated cells were seen, and in one animal (Dog 4) many of the nuclei were hyperchromatic.

In most livers, the Kupffer cells were unusually prominent because of coarse, dark chromatin granules in their nuclei and fine, brown pigment granules in the cytoplasm.

The changes in liver cells described affected the entire parenchyma but were most severe in the portal areas. In some of the central areas there was more intense staining and a few identifiable sinusoids, indicating less rarefaction of cytoplasm and less swelling of the cells.

The livers of all experimental animals stained intensely for glycogen and were not distinguishable, one from another, on this basis. The liver of only one of the control dogs fed on a high protein diet showed any appreciable amount of

glycogen by histochemical examination and even in this instance the amount was estimated to be less than half that found in the experimental animals. It appeared to be equally distributed between the portal and central areas, with considerably less present in the mid-zonal region. In the five normal dogs examined, only occasional patchy areas of glycogen were encountered. The three of these dogs which received chow and meat had been fasted sixteen to twenty hours, but the two receiving the high protein diet were killed at the same interval after feeding as the experimental animals. The significance of this apparent increase in liver glycogen in protein-depleted dogs is unknown.

Although stainable fat was present in appreciable quantities in the cytoplasm of the cells of the bile duct epithelium in the livers of all experimental animals, and to a lesser extent in the control and stock animals, none was found in the liver parenchyma of any animal. It may be concluded that the choline provided in the diet and the methionine fed as such or as protein was sufficient to prevent the development of fatty livers. The morphologic changes described as occurring in the livers of the experimental Dogs 2, 3, 4, 6, and 7 are probably wholly the result of protein deficiency. The accumulation of fat in the livers of protein-deficient dogs described by Elman & Heifitz[30] may be due in part to choline deficiency.

Appetite.—Of the six dogs started in this study, three showed marked failure of appetite toward the end of the study. This undoubtedly has had some effect upon the analytic values obtained, although it is not evident what this effect may be. There is also no certainty that the failure of appetite is specifically due to protein deficiency, although we consider this a likely possibility. The nitrogen intake of the dogs was maintained by tube feeding during this period, but it was impossible to maintain the caloric intake.

DISCUSSION

We have attempted to evaluate the adequacy of a protein level thought to be near a theoretical minimum, which would correspond to about 20 Gm. in an average human adult. Numerous questions still remain unanswered and the study is being continued, but the present results are instructive. For the reasons discussed in the introduction, the animals were partially depleted of protein prior to being given the low level of protein. During this depletion period the data show that from 96 to 230 Gm. of body protein were lost. This was accompanied by a loss of 9.5 to 13.5 Gm. of circulating plasma protein. The percentage of plasma protein was also decreased slightly, but, without prior knowledge of the plasma levels, would be considered to be in the normal range. ***These changes occurred before a minimum level of urinary nitrogen excretion was reached.*** In our opinion the data on urinary nitrogen excretion and plasma proteins alone would not be considered indicative of severe protein depletion.

During the next four to six weeks when egg protein was fed, all of the analytic data indicate that the animals were maintained in this condition. The general average indicates that nitrogen balance was achieved, and the other constituents measured show no consistent deterioration. The changes during

the period on egg protein are summarized in Table III. However, we interpret the microscopic findings, swelling of the liver cells with marked rarefaction of the cytoplasm, as indicating relatively severe liver depletion. Since the animals appeared to have been maintained, this degree of depletion was probably achieved during the period on the nitrogen-free diet rather than while the egg protein was being fed. It is impossible to state how severe this depletion was, or how serious the consequences were for the animal, but the livers of these animals could not be considered normal. Thus we conclude that the animals which had not been depleted to an endogenous or minimum level of nitrogen excretion were nevertheless too severely depleted to be considered normal and that this base line is too severe to be used for studies which are to be interpreted in terms of normal animals. It seems further apparent that a barely detectable loss of plasma protein has reflected relatively severe liver depletion, and that plasma protein determinations are insufficiently sensitive to evaluate such changes.

TABLE III. ANALYTIC DATA OBTAINED AT THE END OF THE DEPLETION PERIOD COMPARED WITH THE VALUES AFTER APPROXIMATELY FOUR WEEKS ON THE DIET CONTAINING 2 MG. NITROGEN PER BASAL CALORIE

DOG		P.V. (C.C.)	P.P. (GM. %)	T.C.A. (GM.)	ALB. (%)	T.C.A. (GM.)	HB. (%)	HC. (%)	T.C.H. (GM.)	B.V. (C.C.)	B. WT. (KG.)
1	A	450	5.74	25.9	2.76	12.4	13.5	39.8	101	747	13.4
	B	480	6.05	29.0	(2.65)	(12.7)	13.0	38.4	101	779	14.3
2	A	476	7.02	33.4	3.66	17.2	12.4	36.6	93	750	13.4
	B	690	6.77	46.7	(3.00)	(20.7)	10.9	32.1	110	1016	14.0
3	A	504	5.76	29.0	(2.20)	(11.1)	14.2	41.8	123	867	12.7
	B	776	5.76	44.7	3.10	24.1	12.6	37.2	155	1235	14.2
4	A	496	5.76	28.7	(2.30)	(11.4)	12.8	37.2	101	789	13.1
	B	514	5.67	29.1	(2.10)	(10.8)	13.4	39.2	112	845	14.8
6	A	360	5.76	20.7	2.42	12.3	18.1	53.4	131	771	10.0
	B	370	5.67	21.1	2.30	8.5	15.0	44.3	101	667	11.0
7	A	300	5.76	17.2	3.53	10.6	15.5	45.7	86	552	6.5
	B	314	5.58	17.7	(2.20)	(6.9)	14.0	41.3	75	535	7.6
Average, all dogs											
	A	431	5.97	25.8	2.96	12.8	14.4	42.4	105	746	11.5
	B	524	5.92	31.4	2.56	13.4	13.1	38.8	109	846	12.6

P.V., Plasma volume; P.P., plasma protein; T.C.P., total circulating proteins; Alb., albumin; T.C.A., total circulating albumin; Hb., hemoglobin; Hc., hematocrit; T.C.H., total circulating hemoglobin; B.V., blood volume.

A, End of depletion; B, thirty days on egg protein.

The figures in parentheses are estimated from determinations obtained a few days before and after the desired date.

The fact that appetite failed in most of these animals is a serious consequence. One cannot rule out possible deficiencies of other dietary factors as the causes of this, but dogs have been maintained upon a similar diet containing purified casein for long periods. It is not impossible that the low protein diets may change an animal's requirement for other nutrients, but until this is shown we feel we must consider the loss of appetite as a symptom of protein deficiency. This has been observed in other studies on dogs to such an extent as to preclude

completion of the work.[37] Frazier and co-workers[38] have emphasized the immediate effect on appetite of acute amino acid deficiencies. It has been suggested that this is a protective mechanism and that the other amino acids may actually be detrimental in the absence of an essential one. It is also possible that caloric intake itself may be detrimental in this sense since this undoubtedly necessitates considerable work on the part of the liver. However, we have the impression from work during the past several years on low protein diets that the dog and rat are hardly comparable with regard to appetite. Although the food intake of protein-deficient rats is reduced, we have seldom seen a complete failure of appetite such as frequently occurs in dogs, and with rats this usually only has been seen with very severely depleted animals. In dogs, on the other hand, this may be the first suggestion that the diet is unfavorable. In this respect the dog is probably more nearly like human beings. Regardless of whether or not the reduction of food intake is considered a protective mechanism, the vicious cycle of mild deficiency → loss of appetite → severe deficiency is indeed vicious if it reaches the stage where the animal fails to eat an adequate diet when offered.

The differences in the response of the plasma proteins to the feeding of protein were interesting. If the time intervals between determinations had been shorter, it seems likely that the responses might have been more uniform. In most of the animals the response was in plasma volume rather than in the per cent of plasma protein. When plasma protein was increased, this generally appeared to be redistributed to other tissues. Thus the priority seems to be for the maintenance of blood volume and for other tissues rather than for an increase in the concentration of the plasma proteins. If one could determine that level of protein intake at which the plasma levels begin to rise, indicating adequate plasma volumes and flow of protein to less essential systems, this might be a true minimum requirement. However, as mentioned earlier, it is not likely that the determination of plasma proteins would be sufficiently sensitive to identify this point with any assurance.

In a previous discussion,[39] one of us has pointed out that the effect of methionine or egg protein supplementation in lowering the excretion of urinary nitrogen appears to be a lowering of urinary nitrogen to endogenous levels without serious nitrogen depletion. Although it is not clear how this is brought about, it is interpretable if on a nitrogen-free diet the chief deficiency is of methionine. The animal appears to break down proteins and waste other amino acids in order to obtain sufficient methionine. This is consistent with the fact that methionine has functions other than protein synthesis.

It is our conclusion from the present work that the common practice of depleting animals to low nitrogen excretion levels prior to studies on nitrogen requirements is not justified. Levels of nitrogen which apparently maintain such animals in nitrogen balance and also maintain plasma proteins and weight within the low normal range may not be adequate, as evidenced by liver pathology and failure of appetite. However, this level of nitrogen may still be adequate to maintain an animal which has not been depleted, especially if methionine is supplied. Studies similar to these but without prior depletion are needed for the answer to this problem.

SUMMARY

In determining nitrogen requirements, it is recognized that some nitrogen depletion is necessary if minimum requirements are to be determined. Various investigators have considered it desirable to deplete animals to a minimum nitrogen excretion if minimum nitrogen requirements are to be determined. In this study 2 mg. of nitrogen (as egg protein) per basal calorie were fed to dogs which were only partially depleted as evidenced by urinary nitrogen levels. Nitrogen balance, plasma protein, plasma albumin and hemoglobin concentrations, plasma volume, thiocyanate space, total circulating plasma protein, total circulating hemoglobin, body weight, liver analyses, and microscopic examination at autopsy were used to evaluate the adequacy of the level of protein fed.

A slight fall in plasma protein and hemoglobin (both as per cent and as total circulating) was observed before minimum nitrogen excretion was reached. After this partial depletion all of the criteria except the last two in the foregoing list indicated that the animals were being maintained in this condition by the level of protein fed. Chemical analyses of the liver indicated considerable but not excessive nitrogen depletion. However, the microscopic examination of the livers revealed swelling of the liver cells, marked rarefaction of the cytoplasm, and loss of basophilic granules (ribonucleic acid). These changes are considered indicative of severe nitrogen depletion. Appetite also failed in most of the animals.

It is therefore concluded that: (1) this level of nitrogen could maintain partially depleted animals insofar as the usual tests for nitrogen metabolism (nitrogen balance, plasma protein concentration) are concerned; (2) the animals were too severely depleted to be considered normal as evidenced by the pathologic changes in the liver and the failure of appetite; (3) the chemical and physiologic tests used are either inappropriate or insufficiently sensitive to determine the degree of nitrogen depletion indicated by liver examination; and (4) further work using nondepleted animals is required to determine if dietary nitrogen fed at the endogenous level of nitrogen excretion will maintain normal animals.

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A METHOD FOR THE DETERMINATION OF PLASMA CATALASE AND THE VALUES OBTAINED IN NORMAL ADULTS

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LOEW,[1] in 1901, demonstrated that there was a characteristic enzyme in all plant and animal cells with the specific action of decomposing hydrogen peroxide into water and oxygen which he named "catalase." In mammals, the erythrocytes, liver and renal cells contain relatively much more catalase than do other tissues. The clinical importance of the catalase activity of whole blood has been shown to be little or none and the changes in health and disease have been shown to parallel the erythrocyte counts, although in general there has seemed to be less of this enzyme in the red cells in the various anemic states.

The catalase content of plasma or serum in human beings has received scant study. Becht,[2] using relatively crude methods, found evidence of slight catalase activity in serum, as did Jolles and Oppenheim.[3] Oppenheimer[4] stated that the low values found in serum probably were the result of red cell destruction, but he did not make clear whether he meant in vitro or in vivo rhexis. Perlmann and Lipmann[5] showed that catalase is found in the various fractions when the proteins of serum or plasma are fractionated.

Description of a clinical method for the determination of catalase activity in serum or plasma has not been found in the literature. Kurokawa[6] presented observations on dog plasma based upon the original method of Jolles.[7] He stated that, in the dog, bleeding had little effect on the plasma catalase activity, but that injection of distilled water, direct hepatic trauma, and hepatic poisons gave increased values.

We have developed, for the determination of catalase in plasma, a relatively simple clinical method which yields results that are reproducible within 5 per cent, most determinations checking within 2 per cent. This method is similar to that of Jolles in principle but it has been modified to increase the sensitivity for the small amounts of catalase which are found in plasma. The procedure is based upon the determination of the amount of hydrogen peroxide decomposed in a given time by a specific dilution of plasma under constant conditions. When a 1:50 dilution of plasma, in a solution containing 0.02N hydrogen peroxide and 0.8 per cent sodium chloride at a pH of 6.8 (0.006M phosphate buffer), is incubated at a temperature of 22 to 23° C. for twenty minutes, the decomposition of the peroxide in each cubic centimeter of the mixture is designated 1 unit of catalase activity. Hydrogen peroxide is measured by the determination of the amount of iodine liberated from iodide (catalyzed by molybdate), conventional thiosulfate titration being used.

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MATERIALS AND SOLUTIONS

1. Powdered heparin. The product of the Connaught Laboratory which contains 110 cat units per milligram is used. This should be finely powdered and tested for catalase activity. We have found no indications of the presence of catalase in the several lots used.

2. Concentrated phosphate buffer and saline. A 0.06M phosphate buffer at a pH of 6.8 is prepared and enough sodium chloride added to make an 8 per cent saline mixture. This is sterilized by boiling and kept under sterile conditions to prevent mold growth which may have catalase activity.

3. Superoxol.

4. A 0.02N hydrogen peroxide buffer and saline solution. This is made by adding about 0.6 c.c. of superoxol to 50 c.c. of the concentrated buffer saline mixture and diluting to 500 cubic centimeters. Ten cubic centimeters of the resulting mixture should require from 20 to 22 c.c. of 0.01N thiosulfate. This solution is made fresh daily and is used within four hours of preparation.

5. A 0.01N thiosulfate solution. This is made by diluting a 0.1N stock solution of sodium thiosulfate.

6. A 10 per cent solution of potassium iodide.

7. A 1 per cent solution of ammonium molybdate.

8. A 5 per cent by volume sulfuric acid solution.

9. A 1 per cent solution of starch.

PROCEDURE

As much finely powdered heparin as can be obtained on the tip of a toothpick is dusted into a 20 c.c. syringe. A like amount of heparin is dusted into a 15 c.c. centrifuge tube which has a paraffin-covered cork. With a 19 gauge needle about 17 c.c. of blood are slowly withdrawn from an arm vein in the usual manner, care being taken that bubbles of air are not sucked through the blood. The needle is removed from the syringe and the blood is allowed to flow gently down the slanting side of the centrifuge tube until the tube is almost full. The cork is inserted and the tube is gently inverted once. The blood is then centrifuged at a moderate speed for fifteen minutes. About half of the plasma is carefully removed and recentrifuged for a like period. Then 0.2 c.c. of plasma in a Kahn pipette is added to 10 c.c. of the hydrogen peroxide buffer mixture and the solution is surged back and forth in the pipette to insure complete removal of all of the plasma. A 120 c.c. Erlenmeyer flask should be used for this mixture. The solution should be at a room temperature of 22 to 23° C.; if the temperature is above or below this, a water bath kept at this temperature must be employed. Twenty minutes later the enzymatic action is stopped by adding 10 c.c. of sulfuric acid solution. A blank is run in an identical manner except that the sulfuric acid solution is added to the peroxide before the plasma is added. Five cubic centimeters of the 10 per cent iodide solution and 3 drops of the solution of ammonium molybdate are added to each flask. After three minutes the liberated iodine is titrated with the thiosulfate solution in the usual manner. The volume, in cubic centimeters, of 0.01N thiosulfate solution of the blank minus that required by the plasma assay, multiplied by 2.5 gives the catalase activity of 1 c.c. of plasma in terms of the units previously defined. If duplicates do not agree within 5 per cent, the test is repeated.

Comment.—It was found that the drawing of bubbles of air through the blood during withdrawal gives high values owing to destruction of erythrocytes.

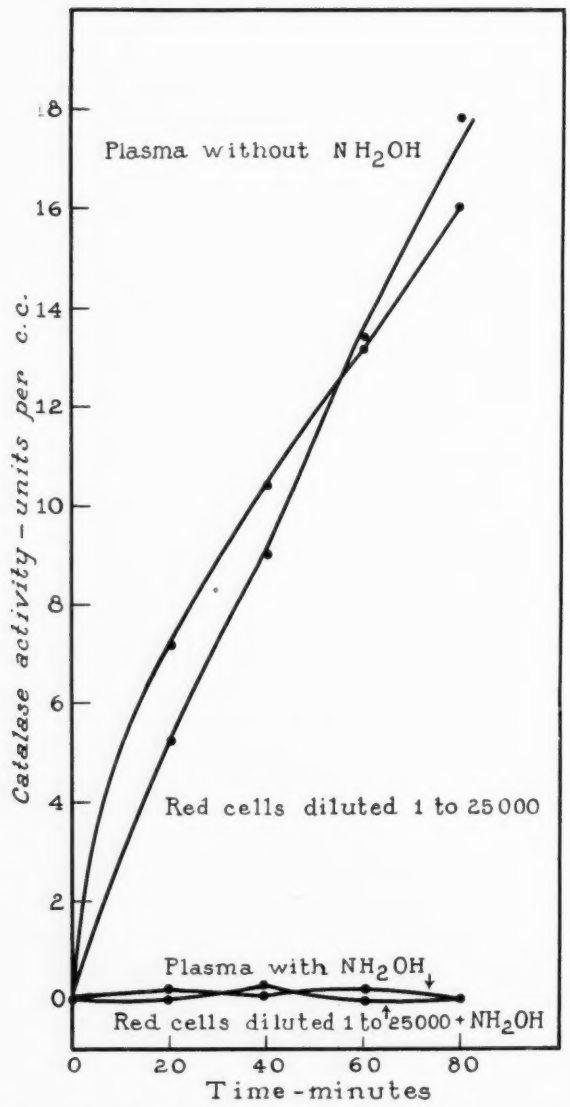

Fig. 1.—The effectiveness of hydroxylamine in inhibiting the catalase activity of lyzed erythrocytes and plasma.

For the same reason it is necessary to allow the blood to flow gently into the centrifuge tube and to avoid squirting. By comparison, it was found that heparin-treated plasma gives lower catalase values than does plasma prepared by the various citrate or oxalate methods. Serum also was found to give much higher values owing to cell destruction during coagulation. Cell-free plasma was found to lose its catalase activity upon standing; in one hour about 10 per cent loss occurs.

That the decomposition of hydrogen peroxide in the procedure just described is due to catalase in the plasma and not to the oxidation, by the peroxide, of the

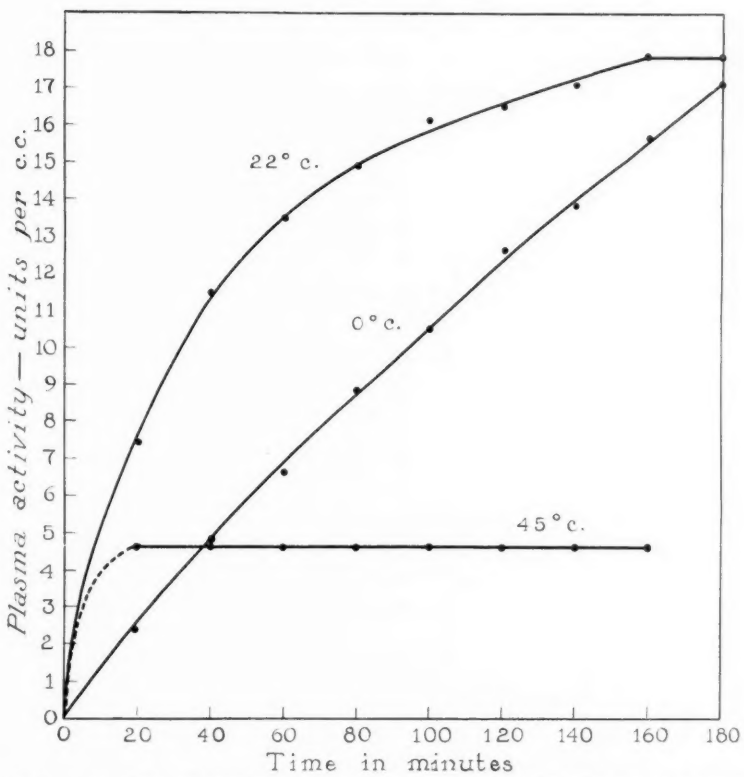

Fig. 2.—The effect of temperature changes on the catalase activity of plasma.

many oxidizable substances known to exist in plasma was shown by various methods. Urine diluted 1:50, with enough glucose added to make a concentration comparable to that of plasma, gives no decomposition of peroxide under the conditions of the present method. It is well known that hydroxylamine is an inhibitor of the action of catalase. As shown in Fig. 1, the presence of hydroxylamine completely inhibited the decomposition of hydrogen peroxide by plasma or by lyzed erythrocytes.

Another proof that catalase activity is responsible for the observed action is the reduction of the maximal amount of decomposition of peroxide with temperature increases. When time and the amount of peroxide conversion are plotted at various temperatures, curves are obtained as in Fig. 2. If non-enzymatic oxidation reactions were involved, one would expect increased activity at higher temperatures, a plateau being reached only when all peroxide was consumed or all oxidizable substances were oxidized. It will be noted that at 45° C. a plateau is quickly reached, but the curves at lower temperatures indicate that this could not be due to lack of available peroxide or oxidizable substances. Likewise, the curve at 22° C. shows a maximum of activity which is lower than the maximum reached at 0° C. The curves are explained by the known peculiarities of catalase. Unlike most enzymes, catalase is rather rapidly inactivated by its substrate. This inactivation is at a minimum at freezing but rapidly increases with relatively small increments in temperature.

The question arises as to whether peroxidase activity may not complicate the estimation of catalase activity. This complication is unimportant, according to the following calculation. If one uses the liberal value for phenols, including tyrosine, epinephrine and bilirubin, in the blood plasma of 10 mg. per 100 c.c. and, using the molecular weight of tyrosine, assumes that one mole of oxygen is used per mole of substrate, it can be calculated that not more than 0.008 mg. of oxygen would be consumed per cubic centimeter of plasma, whereas the oxygen liberated from the hydrogen peroxide by 1 c.c. of plasma, the mean value for the method being used, amounts to 1.12 mg.; hence, less than 1 per cent of the observed value could be ascribed to peroxidase action.

Several other factors indicate that peroxidase activity does not occur under the conditions of the test. No darkening of the reaction mixture, which one might expect if there had been any appreciable oxidation of phenolic compounds, was observed during the test. When plasmas containing much bilirubin—so much that the reaction mixture was a faint yellow—were tested, there was observed no change to the green tint during the enzymatic period which would have been expected if peroxidase activity had been present. It may be remarked that the bilirubin in these instances was oxidized to biliverdin, which gave a green tint, due to oxidation by the free iodine liberated during the assay of the hydrogen peroxide.

It would be difficult to prove that the catalase activity observed was not due to the destruction of erythrocytes during the collection and preparation of the plasma. It can be stated that the method presented gives the lowest values for catalase activity when compared with other methods of preparation of plasma or serum. As will be shown later in another paper on hemolytic diseases, in conditions in which an increase in plasma catalase would be expected, the values for catalase activity are actually much higher than the normal upper limit, but, after correction of the hemolyzing process, the values become more nearly normal. That heparin itself is without effect on the erythrocytes is indicated by the fact that a specimen of blood containing heparin in the amounts specified in this method and another containing about ten times this amount yielded the same results in duplicate.

The use of parafin-lined tubes and oiled syringe gave, in some instances, higher values when compared with heparin duplicates; in other cases closely checking results were found, but in no instance was a value lower than that of the heparin duplicate observed. The possible disruption of erythrocytes during centrifugation was minimized by the use of moderate speeds and by avoidance of rapid increases to top speeds.

A second problem presented itself, namely, whether the catalase activity of the erythrocytes influences the activity as observed in the plasma. This was investigated by making a 1:500 dilution of the erythrocyte layer after centrifuging, a 0.2 per cent solution of sodium carbonate being used as diluent. Then 0.2 c.c. of this lyzed erythrocyte solution was assayed for catalase activity by use of the same method as for plasma except that a thirty-minute digestion period was used. The hemoglobin of the solution was determined by means of

a Photelometer. The amount of hemoglobin expressed in grams per 100 c.c. of erythrocyte layer and divided by the catalase activity of 1 c.c. of the 1:500 solution of lyzed erythrocytes was designated the hemoglobin-catalase coefficient.

RESULTS

The plasma of fifty adults was assayed for catalase activity by the method described. These people were all classed as normal inasmuch as the value for hemoglobin was more than 13.0 Gm. per 100 c.c. of whole blood, and they were considered free of serious disease or disorders in which one would anticipate tissue or blood destruction.

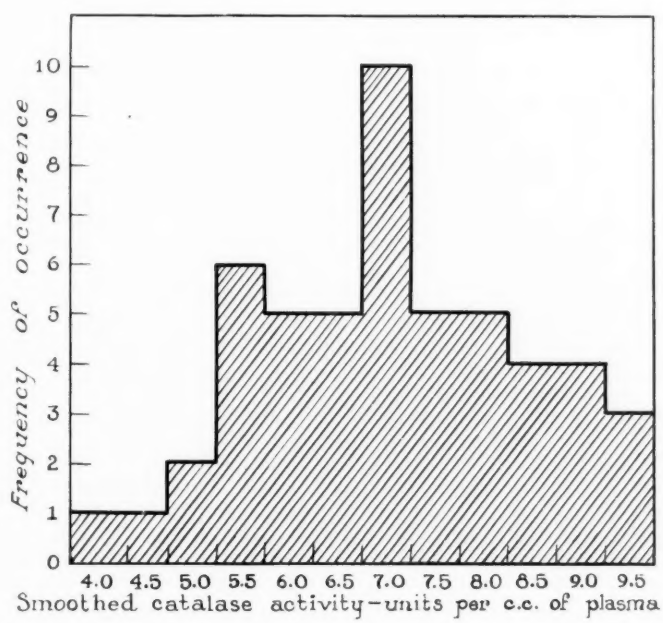

Fig. 3.—Distribution of catalase activity of the plasma in fifty-one normal adults.

Normal values were found to range from 4.2 to 9.5 catalase units per cubic centimeter of plasma; the mean was 6.9 catalase units. When these values were smoothed to the nearest whole or half number, a distribution curve was obtained as shown in Fig. 3.

The hemoglobin-catalase coefficient varied from 2.4 to 3.3 in the twenty-five cases in which it was determined. No correlation was found between this coefficient and the catalase activity of the corresponding plasma, showing that the catalase activity of erythrocytes does not influence the plasma activity.

The daily values for plasma catalase activity in a normal adult over a period of five days were 9.5, 8.5, 9.5, 7.0, and 9.5, respectively. Although but little fluctuation was found in this instance, more determinations on various people are needed before definite conclusions can be drawn.

SUMMARY

A method for the determination of catalase activity of human plasma has been presented. The normal values obtained in fifty adults were found to range from 4.2 to 9.5 catalase units per cubic centimeter of plasma, the mean being 6.9 catalase units. No correlation was apparent between the catalase activity of the erythrocytes and the catalase activity of the corresponding plasma.

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PLASMA CATALASE IN HEMOLYTIC DISEASES AND OTHER ABNORMAL STATES

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A PREVIOUS report[1] has described a clinical method for the determination of plasma catalase and has established normal values for adults. By the use of this method abnormal states have been studied. The procedure has been varied slightly in that with the higher catalase values reached in disease states (with values greater than 50 units, which is the limiting value in the method as described) smaller dilutions of plasma were necessary. Usually 0.2 c.c. of plasma in 50 c.c. of the hydrogen peroxide and buffer mixture was used when there was reason to expect very high catalase values or when values of more than 50 were found using the regular procedure. The catalase coefficient of the erythrocyte layer was determined as described in the preceding paper.

Because erythrocytes, liver cells, and renal parenchyma are relatively rich in catalase, one might theoretically predict some of the known clinical conditions in which an increase in plasma catalase could be found. Any condition in which destruction of erythrocytes was occurring to a greater degree than normally occurs in the blood stream would be expected to give a rise of concentration of catalase in the plasma. Those states in which this might be anticipated include transfusion reactions, congenital and acquired hemolytic anemia, pernicious anemia in severe relapse, acute infections causing rapid anemia, paroxysmal hemoglobinuria, nocturnal hemoglobinuria, and the condition produced by injection of distilled water or other hypotonic solutions. Certain other conditions in which excessive destruction of blood is occurring with release of the contents of the erythrocytes into the blood stream include phlebothrombosis, infarctions of all types (especially myocardial infarctions with formation of large mural thrombi), hemorrhage into the thoracic or peritoneal cavities, and crushing injuries.

Conditions in which there is rapid destruction of hepatic parenchyma, such as acute yellow atrophy, acute hepatitis (infectious), poisoning from the various hepatic toxins, and crushing injury to the liver, would be expected to show some increase in plasma catalase.

There are few renal diseases in which rapid destruction of parenchyma usually occurs, but it is possible that poisoning by the heavy metals might show some change in plasma catalase activity.

We have investigated the plasma catalase in some of the disease states mentioned. Kurokawa[2] had found that in dogs, injections of distilled water, trauma to the liver, and hepatic poisons gave increases in plasma catalase. We were

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TABLE I. VALUES BEFORE, DURING, AND AFTER SPLENECTOMY IN FAMILIAL HEMOLYTIC ANEMIA

CASE	DAYS AFTER OPERATION	HEMOGLOBIN (GM./100 C.C. OF BLOOD)	ERYTHROCYTES (MILLIONS)	RETICULOCYTES (%)	SYSTEMIC VENOUS BLOOD			SPLENIC VENOUS BLOOD			SPLENIC ARTERIAL BLOOD			Hb*/C.A.
					PLASMA CATALASE (UNITS/C.C.)	BILIRUBIN (MG./100 C.C. OF SERUM) DIRECT	BILIRUBIN (MG./100 C.C. OF SERUM) INDIRECT	PLASMA CATALASE (UNITS/C.C.)	BILIRUBIN (MG./100 C.C. OF SERUM) DIRECT	BILIRUBIN (MG./100 C.C. OF SERUM) INDIRECT	PLASMA CATALASE (UNITS/C.C.)	BILIRUBIN (MG./100 C.C. OF SERUM) DIRECT	BILIRUBIN (MG./100 C.C. OF SERUM) INDIRECT	
1	0	4.7	1.0		27.4	1.4	3.9	50+	1.04	1.44	23	0.5	0.8	
	5	7.0			20.0	0.0	0.6							3.4
	16	8.7			15.0	0.0	0.6							2.8
2	Preoperative	14.4	4.7	5.3	10.0	0.0	5.2							3.3
	0							23.7	0.0	6.8	15.8	0.0	5.3	
	8				9.7	0.0	0.3							
3	0	10.8	3.7	18	34.5	5.3	3.5	34.5	4.9	3.1	33	4.9	2.5	
	13	12.9	4.8	0.8	26.0	1.0	0.8							
	17				22.5	0.0	1.2							2.8
	21				17.0									
4	0	12.1	3.9	27	7.5	0.0	3.5	10	0.0	4.3	8.9	0.0	4.1	3.4

$$\frac{*\text{Hb}}{\text{C.A.}} = \frac{\text{Hemoglobin of erythrocyte layer (Gm. per 100 c.c.)}}{\text{Catalase activity of erythrocyte layer per cubic centimeter of 1:500 dilution}}$$

able to investigate plasma catalase in hemolytic anemia, in pernicious anemia, in anemia associated with renal insufficiency, after transurethral resection, and in sundry miscellaneous conditions.

Table I summarizes the results obtained in hemolytic anemia at the time of splenectomy and after the operation. It is of interest that in two cases marked differences were found in the catalase of the blood plasma from the splenic artery and veins, and in these cases noticeable differences were also found in the bilirubin of the serum. In the other two cases only minor differences in both catalase and bilirubin were present. The difference between the plasma catalase of the splenic and systemic veins was probably due to catalase pickup during passage through the liver. The one case in which no difference between splenic vein plasma and systemic vein plasma was found showed considerable direct bilirubin, indicative of hepatic parenchymal disease. This may be the reason for the similar values. As would be expected, there was not much difference in plasma catalase between the splenic arterial blood and the systemic venous blood. These results are a good indication that the method used measures the plasma catalase present in vivo and that the catalase activity found is not due to the destruction of erythrocytes during the collection of the blood and the preparation of the plasma.

The blood plasma catalase activity of systemic venous blood in two cases was within normal limits (although in one case a value of 10 was greater than the highest value found in fifty normal adults). In both cases, however, the anemia was mild and it would seem that the rate of destruction in the spleen was not too great; the liver probably removed most of the catalase produced in the spleen.

The catalase content of the erythrocytes in these cases was essentially normal as determined by the hemoglobin catalase coefficient.

TABLE II. VALUES IN VARIOUS CASES OF HEMOLYTIC ANEMIA

CASE	HEMOGLOBIN (GM./100 C.C. OF BLOOD)	ERYTHROCYTES (MILLIONS)	$\frac{Hb^*}{C.A.}$	CATALASE (UNITS/C.C.)	BILIRUBIN (MG./100 C.C. OF SERUM)		REMARKS
					DIRECT	INDIRECT	
5	6.8	1.5	3.2	36.5	1.3	1.7	Acquired hemolytic anemia; reticuloocyte count 40 per cent
6			2.5	8.5	0.0	1.8	Congenital hemolytic anemia; splenectomy 5 years previously
7	14.6	4.9	3.5	7.7	0.0	0.5	Congenital hemolytic anemia; splenectomy 18 years previously
8	9.4	3.1		31.7	0.0	3.6	Acquired hemolytic anemia
9	3.0	1.0	3.0	37.2	0.7	1.9	Acquired hemolytic anemia; in addition, transfusion reactions following incompatible blood
10	11.9	4.0	3.0	11.6	0.0	1.8	Congenital hemolytic anemia of many years' duration; large firm spleen; reticuloocyte count 5.7 per cent
11	10.8	3.7	3.6	4.8	0.0	2.2	Congenital hemolytic anemia; reticuloocyte count 8.6 per cent

$\frac{*Hb}{C.A.} = \frac{\text{Hemoglobin of erythrocyte layer (Gm. per 100 c.c.)}}{\text{Catalase activity of erythrocyte layer per cubic centimeter of 1:500 dilution}}$

TABLE III. VALUES BEFORE AND DURING TREATMENT OF PERNICIOUS ANEMIA

CASE	DAY OF TREATMENT	RETICULOCYTES (%)	HEMOGLOBIN (GM./100 C.C OF BLOOD)	ERYTHROCYTES (MILLIONS)	BILIRUBIN (MG./100 C.C. OF SERUM) DIRECT	BILIRUBIN (MG./100 C.C. OF SERUM) INDIRECT	PLASMA CATALASE (UNITS/C.C.)	Hb*/C.A.	REMARKS
12	2nd before	1.4	7.3	1.8	0.0	1.7	73	2.5	Treated with 5 units liver extract and 5 mg. folic acid per day
	0	1.4			0.0	1.7	53	2.7	
	4	24.2		1.7	0.0	0.9	12.6		Maximal reticulocyte response on this day
	10	10.0		2.2	0.0	0.4	3.7	2.8	
	19	1.8		3.5			8.0		
	29		11.2	4.0					
13	0	0.9	6.8	1.6			47.6		Treated with 15 units of liver extract per day
	5	20.5		2.0					
	7			2.18			10.2		
	14	7.9		3.1			5.2		
14	0	0.6	4.7		0.0	1.34	36.7		Treated with 15 units of liver extract every day for 16 days
	2	1.7			0.0	1.94	45.2		
	5				0.0	1.87	32.7		
	6	9.5			0.0	1.7	17.1	3.8	
	8	18.0			0.0	1.7	7.8		Maximal reticulocyte response 20.6 per cent on 10th day of treatment
	12	10.6			0.0	1.2	5.2	3.6	
	15	6.6					4.8	3.4	
15	6th before	1.0	8.2		0.0	2.4	46.0		
	0	1.5	8.2		0.0	2.8	43.0	2.8	Treated with 45 units liver extract for first 3 days, then 15 units twice a week
	6	21.7	11.8		0.0	1.4	12.5	2.8	
	9	15.3					9.1		
	14	10.2					9.5		
	19	4.0	11.0				5.8		
16	0	0.4	6.4		0.0	1.6	49.7	2.0	Treated with varying amounts of liver extract and 5 mg. folic acid per day; pneumonia developed on two occasions, 4th to 8th day of treatment and 18th to 22nd day of treatment
	6	28.4	7.3				24.2		
	11	8.6					11.7	2.1	
	15	5.6	10.8				8.1	2.5	
17	0	0.2	6.4	2.1			17.6	3.0	15 units liver on 1st day of treatment and 30 units on 7th day of treatment
	4	13.5					9.0	2.8	
	9	19.5	9.5	3.0			7.1		
18	0	1.4		1.9			21.3	2.1	45 units liver extract 1st and 2nd days of treatment; 15 units on 5th 6th, and 10th days of treatment; regular retic. and R.B.C. not done
	7	4.2		3.2			10.3		
19	0	0.9	8.8	1.9			24.7		Treated with 0.5 unit liver extract and 5 mg. folic acid per day for 25 days
	2	2.9	8.8	1.9			32.5	2.6	
	5	11.1	10.5	1.8			15.0		Maximal reticulocyte response
	7	7.6	10.9	2.6			13.0		
	13	4.6	10.5	3.0			10.0		
	18	0.4	11.5				9.1		
20	0	0.3	6.0	2.06	0.0	1.76	43.0	2.7	
	6	14.0	6.6		0.0	1.0	10.6	2.6	Maximal reticulocyte response
	10	3.8	7.7	1.75	0.0	0.6	5.4	2.4	

TABLE III—CONT'D

CASE	DAY OF TREATMENT	RETICULOCYTES (%)	HEMOGLOBIN (GM./100 C.C. OF BLOOD)	ERYTHROCYTES (MILLIONS)	BILIRUBIN (MG./100 C.C. OF SERUM) DIRECT	BILIRUBIN (MG./100 C.C. OF SERUM) INDIRECT	PLASMA CATALASE (UNITS/C.C.)	Hb*/C.A.	REMARKS
21	0	2.2	6.3	1.03	0.0	2.4	18.9	3.1	15 units of liver extract for 3 consecutive days
	6	18.2	7.7	1.73	0.0	0.9	9.8	2.7	
22	0	0.6	10.5	2.1			13.8		1 unit liver extract and 1 mg. folic acid per day for 30 days
	2	1.5		2.3			15.2	2.6	
	4	7.0		2.9			11.5	2.5	Maximal reticulocyte response
	9	3.9		2.9			14.4	2.3	
	11	1.3		2.9			14.5		
	15	1.1		3.5			12.7		
	21		13.6	3.9			11.5	2.7	
23	0	1.2	8.5	2.2	0.0	0.7	9.7		1 unit liver extract per day for 28 days
	5	5.2	8.2						Maximal reticulocyte response
	10	3.0		2.5			5.8		
	15	2.8	10.0	3.5			7.0		

$$\frac{\text{*Hb}}{\text{C.A.}} = \frac{\text{Hemoglobin of erythrocyte layer (Gm. per 100 c.c.)}}{\text{Catalase activity of erythrocyte layer per cubic centimeter of 1:500 dilution}}$$

The results in patients with hemolytic anemia not operated on or who had undergone splenectomy are presented in Table II. It will be noted that the patients with severe acquired anemia had very high catalase values. The patients with congenital anemias who had undergone splenectomy had normal values. The remaining two patients with congenital hemolytic anemia who were not operated on at this time had only mild anemia with relatively low reticulocyte counts. The plasma catalase activity of systemic venous plasma was a little greater than normal in one case and normal in another; this also was noted in two of the patients with hemolytic anemia in Table I who underwent operation. One can probably assume that, because in these cases the spleen is the organ of destruction and the liver removes catalase from the blood plasma, normal or near normal values would be found in the congenital types of hemolytic anemia except during marked exacerbations or crises; whereas in the acquired types of hemolytic anemia in which the site of blood destruction is more general, possibly in the blood stream itself, higher values will be found.

Again the catalase coefficients of the erythrocyte layer were essentially normal, indicating no great change in the catalase content of the erythrocytes as compared with normal amounts.

Table III presents the catalase and other pertinent values obtained before and during the treatment of pernicious anemia. That an excessive destruction of erythrocytes occurs in severe pernicious anemia is well known not only by the increased indirect serum bilirubin value but also by the increased urobilin and urobilinogen output in the feces. In the days before liver therapy, the finding of excessive hemosiderin at necropsy in cases of pernicious anemia was often noted.

As shown in Table III, all the patients who had pernicious anemia except one showed rather marked increases in the plasma catalase activity. It is also apparent that a prompt fall to normal values occurred coincidentally with the

reticulocyte response. In one case (Case 22) in which the reticulocyte response was not marked, the values fluctuated consistently above normal. Fig. 1 also illustrates the responses obtained in a typical case.

The most plausible explanation for the excessive destruction of erythrocytes in pernicious anemia has been that the poikilocytic and anisocytic cells, which are so numerous, withstand the trauma of circulation more poorly than normal cells. The administration of liver seems to correct the production of these abnormally weak cells immediately; those already present are soon destroyed and the normal rate of destruction of erythrocytes with normal plasma catalase values is quickly established, as is indicated by the cases presented. Although this has been shown previously, using serum bilirubin values during treatment as an index of destruction of erythrocytes, the use of plasma catalase values gives a more direct proof of these rapid changes during liver therapy.

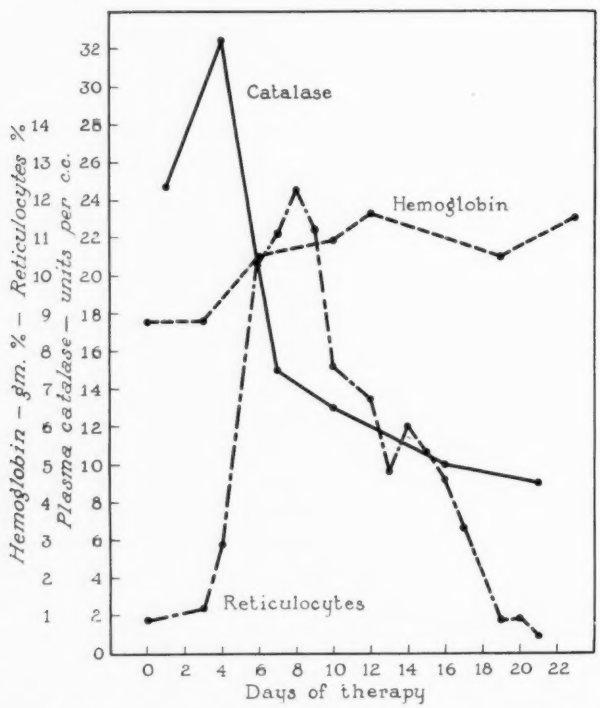
Catalase
Hemoglobin
Reticulocytes
Hemoglobin – gm. % – Reticulocytes %
Plasma catalase – units per c.c.
Days of therapy
0 2 4 6 8 10 12 14 16 18 20 22
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32

Fig. 1.—The effect of liver therapy on the plasma catalase, hemoglobin, and reticulocytes in pernicious anemia.

The catalase coefficients are essentially normal and the changes during treatment do not vary enough to justify considering changes in the catalase content of the erythrocytes during treatment as a determining factor.

The values for plasma catalase found in cases of anemia secondary to renal disease are given in Table IV. The high catalase activity found in many of these cases was an unanticipated finding with no immediate explanation. The bilirubin in these cases was not elevated nor has any previous evidence been found indicating that an excessive rate of destruction of erythrocytes was occurring in such cases of anemia. Some patients were not anemic and none of

them were severely anemic. Because all of them were uremic it was possible that the abnormal amounts of metabolic end products in the plasma were being directly oxidized by the hydrogen peroxide, *but by using hydroxylamine it was shown that these values were due to catalase alone.* It is known that renal parenchymal cells are rich in catalase, but one can hardly explain these values by excessive destruction of renal tissue in the light of present knowledge of chronic renal disease. There was no correlation between the catalase activity in these cases and the blood urea, blood creatinine, or the inorganic constituents of the plasma.

TABLE IV. VALUES IN ANEMIA SECONDARY TO RENAL DISEASE

CASE	HEMOGLOBIN (GM./100 C.C. OF BLOOD)	ERYTHROCYTES (MILLIONS)	PLASMA CATALASE (UNITS/C.C.)	Hb*/C.A.	UREA (MG./100 C.C. OF BLOOD)	BILIRUBIN (MG./100 C.C. OF SERUM)	REMARKS
24	8.6	3.0	More than 50	2.9	140	0.6	D.A.D.,† Group 4, with renal insufficiency and cardiac failure
25	7.6		38.7	3.0	220		Chronic glomerulonephritis; patient died 4 days later
26	13.2	4.3	34.0	2.7	202		D.A.D., Group 4, or chronic glomerulonephritis; differential diagnosis not possible
27	7.1	2.3	33.0	2.4	234	0.5	Chronic glomerulonephritis
28	8.4	3.9	18.0		146	0.3	Chronic glomerulonephritis
29	6.4	3.1	17.7		374	0.4	Chronic glomerulonephritis
30	12.4	4.0	16.5	2.3	78		D.A.D., Group 3, possibly chronic glomerulonephritis; pulmonary edema the previous night
31	10.1	3.9	16.0		156		Chronic glomerulonephritis, possibly D.A.D. with secondary renal insufficiency; death several weeks later
32	7.6	2.8	12.0	2.7	196	0.4	Chronic glomerulonephritis
33	7.8		11.7	3.0	134		Chronic glomerulonephritis
34	13.6		10.2	2.5	104		Kimmelstiel and Wilson's syndrome with diabetes mellitus
35	9.5	2.7	8.5		142		Chronic glomerulonephritis
36	13.1	3.9	8.5	3.0	128		Chronic glomerulonephritis
37	7.8	3.1	8.5	2.5	146		D.A.D. with hypertension, Group 4
38	10.6	2.8	7.9		178		Postsplenectomy Banti's disease, renal insufficiency, cause unknown

*$\frac{Hb}{C.A.} = \frac{\text{Hemoglobin of erythrocyte layer (Gm. per 100 c.c.)}}{\text{Catalase activity of erythrocyte layer per cubic centimeter of 1:500 dilution}}$

†Diffuse arterial disease.

Again the catalase coefficients of the erythrocyte layer are within normal limits, a fact which shows that changes in catalase of erythrocytes were not a deciding factor.

It has been shown previously that hemolysis of blood occurs during prostatectomy.[3] This is probably due to the forcing of the distilled water which

is used to irrigate during the procedure into the venous openings in the prostatic beds. It was considered that the use of our method before and after prostatectomy would be of interest. Table V presents the results obtained. As indicated, in all but one case appreciable, and in many cases marked, increases of plasma catalase were found after prostatectomy. In several cases the plasma was grossly

TABLE V. PLASMA CATALASE LEVELS BEFORE AND AFTER TRANSURETHRAL RESECTION IN WHICH DISTILLED WATER WAS USED AS AN IRRIGATION MEDIUM DURING THE PROCEDURE

CASE	POSTOPERATIVE TIME (HR.)	PLASMA CATALASE (UNITS/C.C.)	REMARKS
39	Preoperative	6.5	
	3	24.0	Plasma normal color
	24	11.5	
	48	11.0	
40	Preoperative	8.8	
	3	188.7	Plasma grossly red
	24	68.7	Plasma light pink
	96	20.5	Plasma normal color
41	Preoperative	4.0	
	1	15.8	
	3	16.5	
	6	10.5	
	24	6.2	
42	Preoperative	4.5	
	3	60.5	Plasma showed tinge of pink
	12	28.5	
	24	12.6	
43	Preoperative	9.5	
	3	174.0	Plasma grossly red
	12	93.5	Plasma light pink
	24	43.2	No apparent color
	48	12.3	
44	Preoperative	7.4	
	3	20.0	
	24	14.5	
	48	10.2	
45	Preoperative	9.5	
	3	19.0	
	12	11.2	
46	Preoperative	7.4	
	3	20.0	
	24	10.6	
47	Preoperative	9.0	
	3	13.2	
48	Preoperative	7.7	
	3	11.2	
49	Preoperative	5.4	
	3	6.8	

hemolytic. We consider these results an additional check on the validity of the method previously presented.[1] We also noted that these increased amounts of plasma catalase were not as rapidly removed as one would expect. Further studies comparing plasma catalase with plasma hemoglobin after prostatectomy would be of interest. We think that this method will be more sensitive than the present plasma hemoglobin procedures for detecting minimal hemolysis.

TABLE VI. PLASMA CATALASE IN SOME MISCELLANEOUS CONDITIONS

CASE	HEMOGLOBIN (GM./100 C.C. OF BLOOD)	ERYTHROCYTES (MILLIONS)	BILIRUBIN (MG./100 C.C. OF SERUM) DIRECT	BILIRUBIN (MG./100 C.C. OF SERUM) INDIRECT	PLASMA CATALASE (UNITS/C.C.)	Hb*/C.A.	DIAGNOSIS AND REMARKS
50	13.5	5.1	11.6	4.4	6.5	2.8	Carcinoma of head of pancreas
51	10.3	3.24	8.2	1.1	6.1	3.2	Chronic hepatitis with ascites of 3 years' duration
52	5.8	1.4			7.0		Aplastic anemia; reticulocytes 0.3 per cent
53	14.2	4.5			4.1		Carcinoma of the stomach
54	10.3	4.2	0.5	0.2	7.8	2.5	Lymphoblastoma, Hodgkin's type
55	10.5	4.9			7.2		Uremia secondary to prostatic obstruction
56	17.0	5.9	0.0	1.0	10.2		Polycythemia secondary to low vital capacity, heart failure, and residence at high altitude (5,000 ft.)
57	9.8	3.3			7.2		Chronic lymphatic leucemia
58	10.9	3.1			4.0		Carcinoma of prostate with metastasis
59	8.0	1.5	0.0	1.16	12.0		Cirrhosis of liver; blood film showed 3 per cent reticulocytes, macrocytosis, increased regeneration
60	3.5	1.0		0.75	11.6	3.0	Chronic hypoplastic anemia; some evidences of excessive destruction of erythrocytes (reticulocytes 78 per cent; stool urobilinogen 106 mg. in 24 hours
61	11.9	3.7	0.0	1.1	42.4		Diagnostic problem: probably chronic mild hemolytic anemia acquired; no spleen; one episode of jaundice; reticulocytes 3.7 per cent; blood film showed increased regeneration and few spherocytes; fragility 0.44 to 0.34 per cent
62	16.3	6.6			13.8	2.5	Polycythemia vera

$$\frac{*Hb}{C.A.} = \frac{\text{Hemoglobin of erythrocyte layer (Gm. per 100 c.c.)}}{\text{Catalase activity of erythrocyte layer per cubic centimeter of 1:500 dilution}}$$

Table VI gives values obtained in some miscellaneous cases. It is of interest that the presence of jaundice per se does not influence the values obtained. There is some indication that the high value found in Case 59 was due to increased destruction of erythrocytes (blood film and indirect bilirubin). Case 61 was of unusual interest. The patient had no splenomegaly or familial history. She presented a story of an attack of jaundice a year previously following sulfonamide therapy which was then considered to be responsible. The blood film, increased indirect bilirubin, and increased catalase values would indicate that excessive destruction of erythrocytes was occurring. That in two cases of polycythemia the concentration of plasma catalase was greater than normal is of interest in that on theoretic grounds some increase would be expected. The catalase coefficient of the erythrocytes in the few cases in which this procedure was done again was within normal limits.

Because of the similarity of the chemical structure of catalase and hemoglobin, it is of additional significance that their ratio does not vary from normal in the various disease states studied.

SUMMARY

A discussion of various disease states in which increased plasma catalase might theoretically be expected has been presented.

Using a method previously described,[1] plasma catalase values have been determined in a number of disease states in which elevated levels might be expected. Elevated levels were found in acquired hemolytic anemias and in some familial hemolytic anemias. Differences in plasma catalase of splenic arterial blood and venous blood were found. Increased plasma catalase values were present in pernicious anemias and rapidly fell to normal with treatment. Increased amounts of catalase were present in the plasma in some cases of chronic renal disease. Moderate to marked plasma catalase activity was found after prostatectomy in which distilled water was used as the irrigating medium. Sundry other diseases in which excessive plasma catalase would not be expected gave normal values. The determination of plasma catalase may be of practical value in diagnostic problems.

The ratio of hemoglobin to catalase did not vary from normal in the disease states studied. Thus, variation in the catalase of erythrocytes was not a determining factor in the changes from normal found in plasma catalase in the cases presented.

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EFFECT OF VARIOUS LETHAL PROCEDURES AND THERMAL INJURY ON CAPILLARIES

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THE role of capillary hyperemia in shock has long been a subject of controversy, but in the past, errors of interpretation have arisen in part because of inadequate methods of tissue examination.[1, 2] One potential source of error was uncovered when it was shown that congestion was often masked in unexsanguinated specimens.[3] If an animal dies from shock and sufficient time is permitted before the organs are removed, it is found that the organs of the shocked animals appear similar to or only slightly more congested than the corresponding organs of nonshocked animals. On the other hand, if the animals are killed by exsanguination, the organs of shocked animals are much more congested than the organs of the unshocked animals. Similarly, when organs are removed in vivo, the difference in the amount of congestion in the shocked and unshocked animals becomes manifest only after the extirpated organs are permitted to bleed out.

There is a second potential source of error which may invalidate many previous studies on congestion in shock. The agent used to kill the experimental animal may in itself be responsible for a considerable amount of congestion. In this instance fallacious conclusions would be drawn if the organs of an animal dead of shock were compared with the organs of a nonshocked animal destroyed by a lethal dose of anesthetic.[4]

The method employed in this laboratory for estimating the degree of congestion is a comparison of the amount of hemoglobin and the number of capillaries in the exsanguinated organs of shocked and nonshocked animals. The procedures for making hemoglobin assays[5] and capillary counts with the use of special stains for the erythrocytes[3] have been previously described.

If these possible sources of error are taken into account, it becomes evident why a pathologist who examines the tissues in the usual way is unable to determine with precision the amount of congestion in a given organ at autopsy. In the first place, he has no opportunity to estimate the amount of congestion by comparing exsanguinated organs. Second, he does not make hemoglobin assays and capillary counts. Third, there is a possibility that terminal capillary atony occurs in death from a variety of causes other than shock.

It is the latter possibility that requires further investigation, and to this end experiments were performed with a variety of lethal agents to determine to what extent they are capable of producing capillary hyperemia.

Experiment.—The object of the following experiment was to ascertain the amount of congestion which develops in a test visceral organ, the kidney,

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after a number of designated lethal procedures. A separate group of animals was subjected to fatal thermal trauma in order to compare the amount of congestion in a classic form of shock with that in the first group.

Forty-two Long-Evans rats weighing from 153 to 374 grams were divided into seven groups of six animals each and treated in the following manner. Under local anesthesia, with procaine, the right renal pedicle of each animal was tied and the kidney removed and allowed to bleed freely. The animals were then killed by various means. In the first five groups, death was produced by sodium pentobarbital (0.4 c.c. intravenously), procaine (0.4 c.c. intravenously), sodium cyanide (8 mg. intravenously), ether (by inhalation), and asphyxia (by clamping the trachea), respectively. In the sixth group, death was caused by a severe burn (immersion to the thorax at 100° C. for thirty seconds). In the seventh group, death was produced by rapid exsanguination from the abdominal aorta.

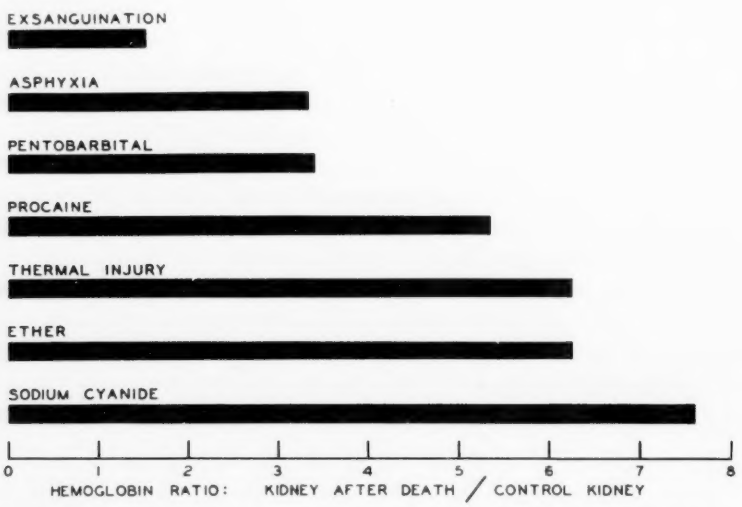


Fig. 1.—Relative amounts of hemoglobin retained in the exsanguinated kidneys of rats subjected to various lethal procedures. The control kidney was removed first, under local anesthesia. After death, the second kidney was removed and the hemoglobin content compared with the control. The pooled kidneys of six animals were used in each test.

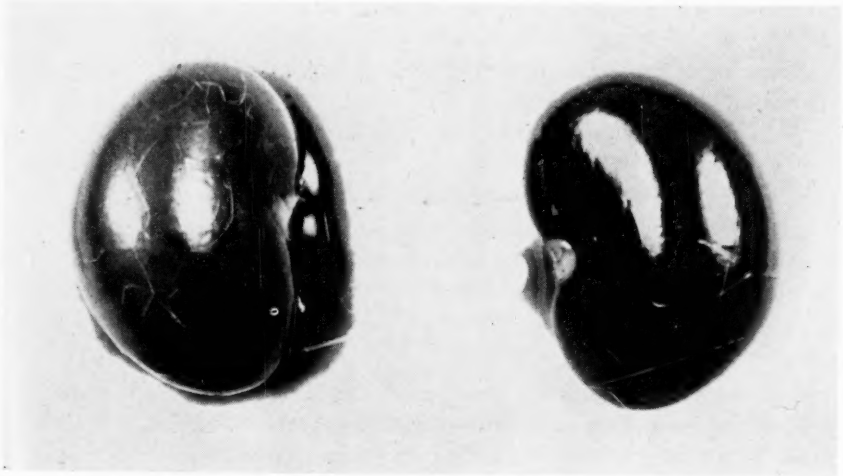
The latter group served as a further control. Twenty minutes after death, in each instance, the second kidney was removed and allowed to bleed out. The hemoglobin content of both kidneys of each animal was then ascertained.

Results.—In every instance the second kidney contained a significantly greater amount of hemoglobin than the kidney removed before the animal was killed. In the seventh group, in which death was by exsanguination only, the increase was a small one. The findings are presented in Fig. 1 in terms of the ratio of hemoglobin in the kidney removed after death to that in the control kidney.

This experiment shows that various lethal procedures as well as burns cause congestion in a test visceral organ and that the amount of congestion is quantitatively of the same order as that which develops in fatal burn shock.

A. *B.*

Fig. 2.—Comparison of kidneys before and after a fatal burn. One kidney (*A*) was removed immediately before scalding of the body up to the head at 100° C. for two minutes and is the control for the second kidney (*B*) which was removed three minutes after the burn. Both kidneys were exsanguinated after removal. Note the dark, engorged appearance of the second kidney, with little blood around it, and the pool of blood around the pedicle of the first kidney, after exsanguination.



A. *B.*

Fig. 3.—Comparison of kidneys before and after a lethal dose of ether. One kidney (*A*) was removed immediately before administering ether and is the control for the second kidney (*B*) which was removed after fatal etherization. Both kidneys were allowed to self-exsanguinate. Note the dark, engorged appearance of the second kidney and the pool of blood around the pedicle of the first kidney.

This experiment confirms previous observations that the degree of congestion can best be demonstrated by making comparative examinations on exsanguinated organs.

In order to illustrate pictorially the effect upon the kidneys of a severe burn and a lethal dose of ether, respectively, the following experiments were performed. Two animals were used. In each instance, one kidney was removed

under light ether anesthesia and allowed to bleed out. The first animal was killed with ether, and the second by a severe burn. The second kidney of each animal was then removed and allowed to bleed out. Kodachromes were taken of the two sets of kidneys (Figs. 2 and 3). The engorgement of the second kidney in each instance is striking.

DISCUSSION

If an animal dies from shock and sufficient time is permitted before the organs are removed, it is found that the organs of the shocked animal appear similar to, or only slightly more congested than, the organs of a nonshocked animal. In previous communications it was shown that the difference between the gross appearance of the organs of shocked and nonshocked animals becomes manifest if the animals are killed by exsanguination.[3] Obviously when tissues are taken for study some time after death has occurred, it is no longer possible to make such a comparison. The importance of exsanguination before examining the tissues in shock may explain previous failures to appreciate fully the vascular factor in shock.

In the present report it was shown that asphyxia, lethal doses of pentobarbital, procaine, sodium cyanide, and ether cause visceral congestion. In each instance the hemoglobin content of the kidney removed just prior to the administration of the fatal dose and allowed to bleed out was compared with that of the second kidney removed and treated similarly twenty minutes after death. When the two kidneys were similarly compared in the instance of death from exsanguination, it was found that the hemoglobin content of the second kidney was greater than the first, but that the amount of increase was much smaller than in the other experiments. The operative procedure involved in the removal of the first kidney causes a certain amount of trauma, and this could explain why in the exsanguination experiment the second kidney contained more blood than the first. In the experiments with the other lethal procedures, the difference in the hemoglobin content of the two kidneys was striking. Over seven times as much blood was found in the kidneys of the animals killed with cyanide.

These observations clearly show that capillary atony leading to visceral congestion develops from a wide variety of causes. The list includes burns, asphyxia, lethal doses of various drugs, and muscle-crushing injury[6]; it is highly probable that death from many other causes is accompanied by capillary congestion. In determining whether visceral congestion is present in fatal shock, it would be a mistake to compare the degree of congestion in organs of animals in shock with that in corresponding organs of control animals sacrificed by a lethal dose of a substance like pentobarbital or ether. The utilization of these agents for the sacrifice of control, nonshocked animals may explain why many investigators have failed to find or have denied the existence of capillary congestion in shock. Mild or moderate anesthetic doses of these drugs do not cause a significant degree of visceral congestion,[7] but, as was demonstrated, lethal amounts do.

Since visceral congestion, if sufficient in degree, leads to a reduction in the circulating blood volume or the venous return, the development of capillary congestion after lethal doses of certain chemical substances (pentobarbital, procaine, sodium cyanide, and ether) and asphyxia suggests that the engorgement of the viscera may play an important role in the mechanism of death. As was demonstrated, the amount of congestion was fully as great in some instances as after thermal trauma. This raises the question of whether a shocklike process is not implicated in the mechanism of death after the administration of the chemicals investigated.

It may be possible to go a step further. Death from many causes may be accompanied by a significant degree of visceral congestion which the pathologist is unable to evaluate because of the factors mentioned. If the human heart is removed immediately after death from a large variety of causes, it may, with perfusion, be made to beat again, and this would indicate that the immediate cause of death was not failure of the heart. Is it not possible that, in a wide variety of preagonal states due to infections, intoxications, and so forth, the sequestration of blood in atonic visceral capillaries may initiate a shocklike process which constitutes the final disorder of function which precedes and is the final cause of death?

SUMMARY

It was found that asphyxia and lethal doses of pentobarbital, ether, procaine, and sodium cyanide cause visceral hyperemia in a degree comparable in some instances to that which develops after fatal thermal trauma.

When a pathologist is called upon to decide whether or not hyperemia is present in the viscera of a shocked organism, he should bear in mind the results of comparative examination of organs after exsanguination and the fact that visceral congestion may be present after death from many causes other than classic shock, especially in organs of control animals sacrificed by lethal doses of anesthetic and other compounds.

It is suggested that the failure of some investigators to find unusual capillary hyperemia in shock is due to the visceral hyperemia which they have observed in control or nonshocked animals sacrificed by methods which in themselves cause intense visceral hyperemia.

We wish to extend our thanks to Dr. Ben Sacks for valuable aid in the preparation of this manuscript.

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THE MECHANISM OF BACTERIA-INDUCED SHOCK RESULTING FROM CRUSHING OF MUSCLE IN DOGS

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IN PREVIOUS studies, shock was induced in dogs by the crushing and replacement of surgically excised muscle. It was demonstrated that bacterial contamination of the traumatized muscle was responsible for the shock state, because, if bacterial growth was inhibited by suitable antibacterial agents, shock was prevented.[1] The amount of edema in the traumatized parts was too small to account for the development of shock.

In a series of experiments in rats and mice it has been shown that visceral congestion, due to atony of the vessels comprising the capillary bed, is a major factor in the initiation of the shock syndrome which develops after thermal trauma.[2] This factor operates by sequestration of blood in the dilated and atonic vessels of the visceral organs, which is demonstrable in exsanguinated animals or organs,[2] as a result of which the effective circulatory volume and the venous return fall to shock levels. In effect, the animal bleeds into its own capillary bed. In certain circumstances, local fluid loss is also a factor.

The purpose of the present investigation was to ascertain whether the factors operative in shock due to muscle crushing[1] are the same as those in thermal shock. Accordingly, experiments were conducted to determine whether the toxic factor from crushed infected muscle leads to capillary atony and a reduction in bleeding volume.

METHODS

The quadriceps femoris of dogs anesthetized with sodium pentobarbital was removed from one hind limb. The muscle was cut into fine pieces, ground in a sterile mortar, and replaced in its original bed. The amount of muscle crushed corresponded to 3 to 5 Gm. per kilogram of body weight. Sterile precautions were observed throughout Experiment 1. As described in previous reports,[1, 3] animals subjected to this procedure were obviously in shock at the end of twenty-four hours. There was a decrease in the circulatory volume; the limb operated upon was edematous; and, despite the sterile technique, the crushed muscle at autopsy always had a foul odor, gas was often present, and direct smear of the muscle revealed numerous bacteria of many types.

Bleeding Volume.—Under sodium pentobarbital anesthesia the femoral artery was cannulated and the blood received in a weighed container until exsanguination was complete. The bleeding volume is expressed as the weight of blood obtained as a per cent of the body weight.

Hemoglobin Determination.—The amount of hemoglobin retained in two test organs, the kidney and the liver, after exsanguination of the animal was used as an index of the degree of capillary atony in the viscera. Hemoglobin was extracted from the finely cut organs with 20 per cent urea solution and readings were made on the Fisher Electro-Hemometer. A more satisfactory method of determining hemoglobin in tissues has been developed subsequently and is described in detail in another publication.[4]

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Histologic Examination.—The method of staining the tissues to facilitate the identification of the erythrocytes and the procedure for making capillary counts are described in a previous publication.[2]

Experiment 1.—Under sodium pentobarbital anesthesia, twelve dogs weighing from 7.8 to 17.2 kilograms were subjected to the crushed muscle procedure. Upon the development of profound shock (twenty to forty hours after the operation), each animal was reanesthetized and exsanguinated, and the bleeding volume was determined. Sections of various organs (heart, liver, kidneys, spleen, adrenals, intestines, and lungs) were taken for histologic study, and hemoglobin determinations were made of the liver and kidneys of each animal. For comparison, an equal number of normal animals was exsanguinated and investigated in the same manner.

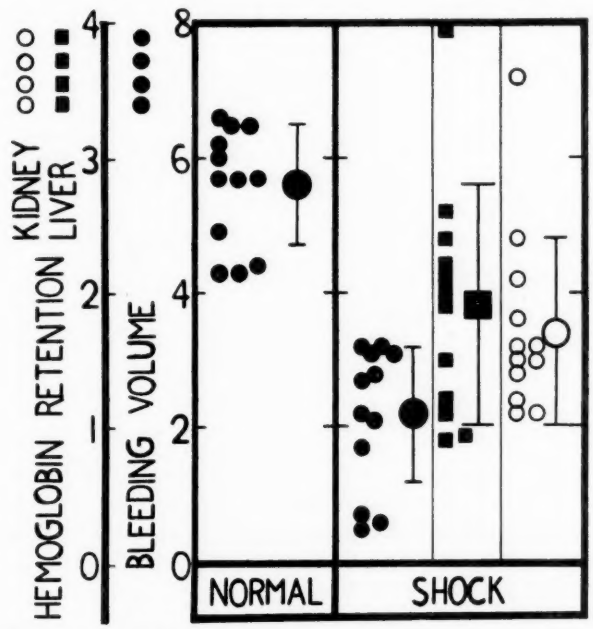

Fig. 1.—Graph showing relation of bleeding volume and hemoglobin retention in the liver and kidneys of dogs in shock produced by muscle crushing. Bleeding volume is expressed as the per cent of blood weight to body weight. Hemoglobin retention is in arbitrary units based on retention by normal tissue as unity. The large dots, square, and circle represent the average of the values graphed. The verticle lines through these points are equal in length to 2 times the standard deviation and the horizontal lines above and below these represent the standard error of the mean.

In the shock experiments, bleeding volume was significantly reduced and retention of blood in the liver and kidneys after exsanguination was significantly increased.

Results: The bleeding volumes of the shocked animals ranged from 0.53 to 3.2 per cent of body weight (mean, 2.2 per cent), and those of the control normal animals from 4.3 to 6.6 per cent (mean, 5.6 per cent) (Fig. 1). These values for the bleeding volume in normal and shocked dogs are similar to those reported by other investigators.[5] The hemoglobin content of the kidneys and liver of shocked animals was considerably greater than that of the corresponding organs of normal control dogs (Fig. 1). Histologic examination revealed that the number of open capillaries in the organs of shocked animals was increased

and that the capillaries were wider and contained more erythrocytes than the corresponding organs of normal control animals. The examiner, without knowing which particular slide he was studying, was able in each instance to identify the organs of the shocked animals by the appearance of the capillaries.

In this experiment the physiologic changes which accompany shock, that is, visceral congestion and reduction in bleeding volume, are similar to those demonstrated in mice and rats after thermal trauma. In both instances a circulating chemical factor is responsible for the observed capillary atony. In the muscle-crushing experiment, this factor, a product of bacterial contamination, may be a bacterial toxin.[6-9]

Experiment 2.—The following experiment was performed on four dogs in which each animal served as its own control. Under sodium pentobarbital anesthesia the left kidney of each animal was removed and the organ allowed to self-exsanguinate for twenty minutes. After exsanguination was complete, it was weighed, sections were taken for histologic study, and the hemoglobin content was determined. The muscle-crushing procedure was carried out immediately after nephrectomy. Approximately thirty hours later, while the animal was in profound shock, the opposite kidney was removed under sodium pentobarbital anesthesia and investigated in the same manner as the control kidney. The bleeding volume was measured immediately after the removal of the second kidney.

Results: In each instance the kidney removed after the animal had entered into shock was more congested than the control kidney; it contained, on an average, more than twice as much blood as the normal kidney; and histologic study showed a large increase in the number of open capillaries, together with an increase in the diameter of and the amount of blood in every capillary. In three of the shocked animals the kidney weighed from 5.4 to 13.3 per cent more than the normal control kidney, and in the fourth animal the weights were the same, although the second kidney was more congested than the first. The bleeding volume in the shocked animals varied between 1.8 and 2.6 per cent of the body weight. This experiment, like the previous one, shows that a major factor causing the decreased bleeding volume in shock after muscle crushing is congestion of the viscereal organs resulting from atony of the vessels comprising their capillary beds.

DISCUSSION

The results of Experiments 1 and 2 clearly indicate that shock due to muscle crushing results from a reduction in the effective circulatory volume and that a principal cause of this reduction is visceral congestion due to capillary atony. Thus, the shock-producing mechanism in crushed muscle injuries is similar to that observed in burns, except that in the former instance the toxic factor which leads to capillary atony is derived from infected muscle tissue. Shock induced by thermal injury in mice is due to a toxic factor not of bacterial origin because the shocked state may occur within a few minutes after severe injury.[2] Furthermore, the administration of antibacterial substances has no effect on the mortality after thermal injury.[10, 11]

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THE MECHANISM OF DELAYED DEATH FOLLOWING THERMAL TRAUMA

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IN PREVIOUS observations it was shown that burn shock was accompanied by a reduction in the circulatory blood volume, as represented by the bleeding volume, and that two major factors were implicated in this reduction: one, local fluid loss, and the other, atony of the vessels comprising the capillary bed of the visceral organs.[1] One of the methods employed for estimating the degree of capillary atony was measurement of the amount of hemoglobin retained in a test organ. In previous experiments it was shown that after a severe and fatal burn there is a progressive diminution in the bleeding volume, whereas after a less extensive burn there is an initial decrease followed by a gradual increase in bleeding volume as the animal recovers.[1]

In view of the fact that most of the previous observations were not carried beyond a twenty-four hour period,[1] it was considered of interest to ascertain whether any of the animals would succumb after apparent recovery from the initial period of shock. Observations showed that a number of animals died at various time intervals, from the second day to more than a month later, and an investigation was undertaken to determine the bleeding volume and degree of capillary atony in surviving animals in an endeavor to elucidate the mechanism of delayed death after thermal trauma.

METHODS

The bleeding volume[1] was ascertained by cutting out the heart of the etherized animal and mopping up the blood entering the thoracic cavity with weighed cotton pledgets. The presence and degree of capillary atony were determined by measuring the amount of hemoglobin retained in the liver. Briefly, the method consists of extracting the hemoglobin from the finely cut liver with a buffered salt solution and measuring it photometrically after conversion to cyanmethemoglobin. The tissue hemoglobin values were corrected for polycythemia and anemia, respectively. Other details of the method have been described elsewhere.[1, 2]

All animals were placed upon a diet of Alber's Friskies, with the daily addition of 10 mg. of ferrous sulfate, 0.2 mg. of manganese chloride, and trace amounts of cobalt and copper salts per 100 ml. of drinking water. These salts were added in an effort to counteract the anemia which was found to develop after the initial period of hemoconcentration in burned rats. In previous observations it had been shown that this diet with the salts added did not influence the bleeding volume.

Experiment 1: Bleeding Volume and Hemoglobin Retention in Mice at Various Time Intervals After Thermal Injury.—Each of 412 adult male Swiss mice was anesthetized with ether and immersed up to the head in water at 60° C. for seven seconds. The type of burn chosen was one which would permit most of the animals to survive for days or weeks after the initial period of shock. At intervals of two to eight hours during the first twenty-four hours, at the end of two and four days, respectively, and thereafter at stated intervals, a group of surviving mice were etherized and the bleeding volume and amount

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of hemoglobin retention in the liver ascertained. Upon each occasion when these determinations were made, control observations were taken upon a group of normal, unburned mice. The number of animals in the burned group which died each day was recorded and a mortality curve constructed (Fig. 1).

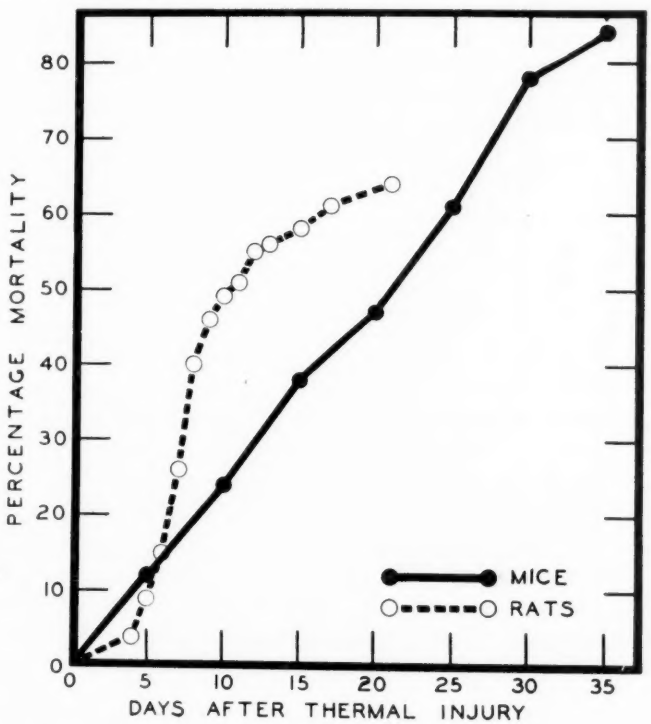

Fig. 1.—Time-mortality curve based on (1) 300 mice scalded to the head in water at 60° C for seven seconds, and (2) 80 rats scalded to the head in water at 65° C. for ten seconds.

Results: The changes in the bleeding volume and the amount of hemoglobin retention in the liver are shown in Figs. 2 and 3. The greatest decline in the bleeding volume was noted in animals which were sacrificed four hours after the burn, at which time the value fell to an average of 2.89 ± 0.16* per cent of body weight, as compared with the average control figure of 5.86 ± 0.10.* It should be noted that the relatively mild type of burn employed in this experiment does not lead to the severest form of shock.[3] By the end of twenty-four hours the bleeding volume was back to almost normal. At the end of two hours the amount of hemoglobin retained in the liver was an average of 1.90 times that in the control, and at the end of four hours, 1.54. By the end of ten hours there was no longer an increased hemoglobin retention, showing that the toxic vascular factor was no longer operative. In the subsequent observations, ending thirty-seven days after the experiment was begun, the figures for the bleeding volume returned to normal and there were no further increases above normal in the amount of the hemoglobin retention. Many of the values for hemoglobin retained in the liver fell below normal after the first day. Most of the animals became anemic and all the animals lost weight. A number which died during

*Standard error of the mean.

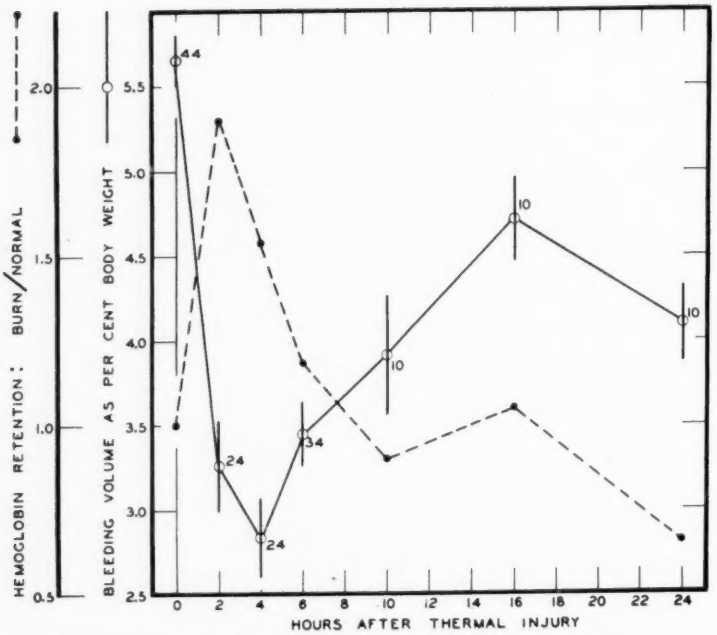

Fig. 2.—Relation of bleeding volume and hemoglobin retention to time after thermal injury (initial twenty-four hour period). Based on 109 mice which were immersed up to the head in water at 60° C. for seven seconds. The verticle lines through the circles have a total length equal to three times the standard error of the mean. The numbers next to the circles represent the number of mice averaged for each point. For the hemoglobin retention graphs of this figure and Fig. 3, the tissue hemoglobin of the burned animals was corrected by the ratio of the hematocrits of normal to burned animals. Within four hours after thermal injury the bleeding volume fell to a minimum value and the hemoglobin retained by the liver had already reached a maximum.

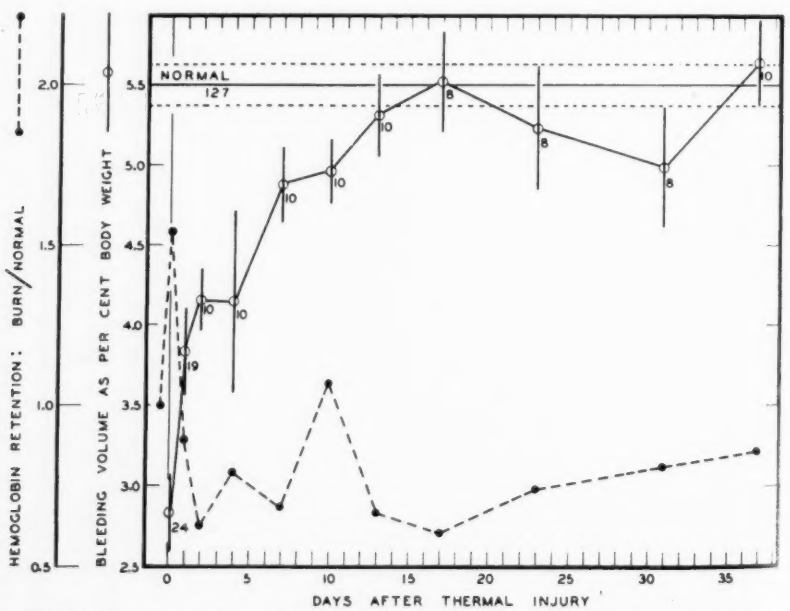

Fig. 3.—Relation of bleeding volume and hemoglobin retention to time after thermal injury (over a period of thirty-seven days). Based on 212 mice subjected to thermal injury. This is an extension of Fig. 2 (the symbols are explained in the text of Fig. 2). The dotted lines accompanying the normal curve for the bleeding volume are separated by a distance equal to three times the standard error of the mean. During the thirty-seven days after thermal injury the bleeding volume returned to normal in about thirteen days, although the amount of hemoglobin retained by the liver appeared to be somewhat less than normal in the burned mice.

the observation period showed infection or gangrene, or both, in one or more limbs. The mode of death was in sharp contrast to that of animals which succumb to burn shock. In the latter instance, the skin temperature falls, dyspnea develops, and death occurs after a period of progressive asthenia and stupor. In the foregoing experiment, many of the animals appeared to be in good condition until very shortly before death and none died with the classic symptoms of shock.

Experiment 2: Rate of Mortality and Bleeding Volume After Thermal Injury in Rats.—A group of 80 Long-Evans rats, weighing from 250 to 400 grams, were anesthetized with ether and immersed in water at 65° C. up to the head for ten seconds. This group together with an equal number of normal unburned rats was placed on the diet described under methods. The animals were observed for twenty-six days. A mortality record was kept and a time-mortality curve constructed. On the twenty-sixth day, all the surviving rats were anesthetized with ether and the bleeding volume was determined. Control observations were made upon an equal number of unburned animals.

Results: In the rats which had survived for twenty-six days, the average bleeding volume was 4.94 ± 0.09* in the controls. Statistical analysis shows that the amount of increase over the control is significant.

A few of the rats developed infection and gangrene of one or more limbs. As in the instance of the burned mice, a number of rats which succumbed at various time intervals after recovery from the initial period of shock appeared in good condition until shortly before death. The time-mortality curve is given in Fig. 1.

DISCUSSION

These observations indicate that in mice which survive burn shock the bleeding volume returns to normal after twenty-four hours, confirming previous studies with this type of burn.[1] Moreover, there was no increased retention of blood in the liver by the end of ten hours, suggesting that the toxic factor which causes atony of the vessels of the capillary bed was no longer operative. As the mortality curve (Fig. 1) shows, a certain number of mice died from two days to more than thirty days after the trauma. Surviving animals were selected at random for sacrifice on a particular day for determinations of the bleeding volume, but in order to ascertain whether the values in moribund animals differed from those in nonmoribund animals, a number of the former were tested at the same time. The values did not differ significantly in the two groups. Neither decreased blood volume nor evidence of capillary atony was demonstrated in any of the animals which survived beyond the initial twenty-four hour period.

Wilson and co-workers[4] followed the clinical course of treated burns in human beings and attributed the delayed deaths in their series to the presence of acute or septic toxemia, rather than to shock. The experiments reported in the present communication clearly show that delayed death in burned mice is not due to shock in the sense of a syndrome accompanied by decreased circulating blood volume and capillary atony.

Whereas the values for the bleeding volume returned to normal in the experiments on mice, the values were found to be above normal in rats which sur-

*Standard error of the mean.

vived twenty-six days after thermal trauma. Noble and Collip,[5, 6] who produced shock in rats by traumatizing them in a specially constructed drum, showed that animals which had been previously subjected to this form of trauma were able to withstand a second trial without being thrown into shock. These authors were unable to explain the immunity which had developed. If an increased blood volume were to be found in these animals, it would help to explain the immunity in the Noble-Collip experiments.

In the absence of visceral congestion and a decrease in the circulating volume, it is necessary to search for causes other than shock to explain the delayed mortality after thermal trauma. Surviving animals develop anemia and loss of weight and not infrequently infection or gangrene of the limbs. Whether the fatal outcome is a result of infection, a metabolic or nutritional disturbance, or is due to some other undetermined cause remains for future studies to explore.

SUMMARY AND CONCLUSIONS

The mechanism of delayed death in burned animals was investigated. The type of thermal trauma chosen for the experiment was one which produced shock but which enabled a large number of animals to survive for a number of days or weeks after the initial period of shock. Observations were made upon the circulatory blood volume, as represented by the bleeding volume, and on the degree of capillary atony, as represented by the amount of hemoglobin retained in the exsanguinated liver. These observations were begun two hours after the burn and continued for a period of thirty-seven days.

In mice, capillary atony and a decreased blood volume were demonstrated during the period in which the symptoms of shock were present. Upon the subsidence of these symptoms (generally within twenty-four hours), the blood volume was restored to its normal value, the capillary atony disappeared, and there was no further reduction in the blood volume or reappearance of visceral congestion throughout the period of observation.

In experiments on rats, after similar thermal trauma, the bleeding volume showed a small but significant increase above the normal in observations made twenty-seven days after the burn. A possible explanation for the immunity to shock in the Noble-Collip experiment was suggested.

Delayed death after the type of thermal injury employed is not due to persistent or recurrent shock.

We wish to extend our thanks to Dr. Ben Sacks for valuable aid in the preparation of this manuscript.

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LABORATORY METHODS

A SIMPLIFIED SEDIMENTATION RATE TECHNIQUE WITH COMBINED CHART AND CORRECTION NOMOGRAM

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THIS paper describes a sedimentation chart and technique that permit correction for the effect of variations in red blood cell-plasma ratio on the sedimentation rate. The method of correction, by means of a nomogram printed directly on the sedimentation chart, is a simplified adaptation of that of Rourke and Ernstene.[1] The sedimentation chart (Fig. 1) is designed for inclusion in the patient's permanent record.

PRINCIPLE OF METHOD

The sedimentation curve of uncoagulated blood, obtained by plotting against time the settling of the topmost layer of cells in a vertical tube, is typically sigmoid and may be divided into three components: (1) an initial slow phase or period of aggregation, during which rouleau formation takes place and the rate of fall gradually increases; (2) a period of constant and maximum rate of fall, represented by a straight line; (3) a period of packing, in which the rate becomes progressively slower as the cells pile up on the bottom of the tube. Sedimentation rate is sometimes defined as the total distance of settling in an arbitrary length of time, usually one hour. This almost always implies a summation of the first two or all three of the phases of sedimentation into a single value, which may be the same for entirely different curves. The unambiguous definition of sedimentation rate as the maximum rate of fall, expressed by the slope of the second, straight-line portion of the curve, has been adopted here.

It is possible to distinguish an elevation in sedimentation rate due to pathologic increase in certain constituents of the plasma, notably fibrinogen, from that brought about simply by anemia. Such a differentiation is customarily made by expressing the sedimentation rate of a blood sample as that which would be observed at an arbitrary, standard packed cell volume; the effect of variations in the cell-plasma volume relationship is thereby eliminated. The two most frequently employed techniques involving a correction of this sort are those of Rourke and Ernstene[1] and Wintrobe and Landsberg.[2] In the latter method the number of millimeters of sedimentation in one hour is corrected for variations from a packed cell volume of 42 or 47 per cent, the average values for

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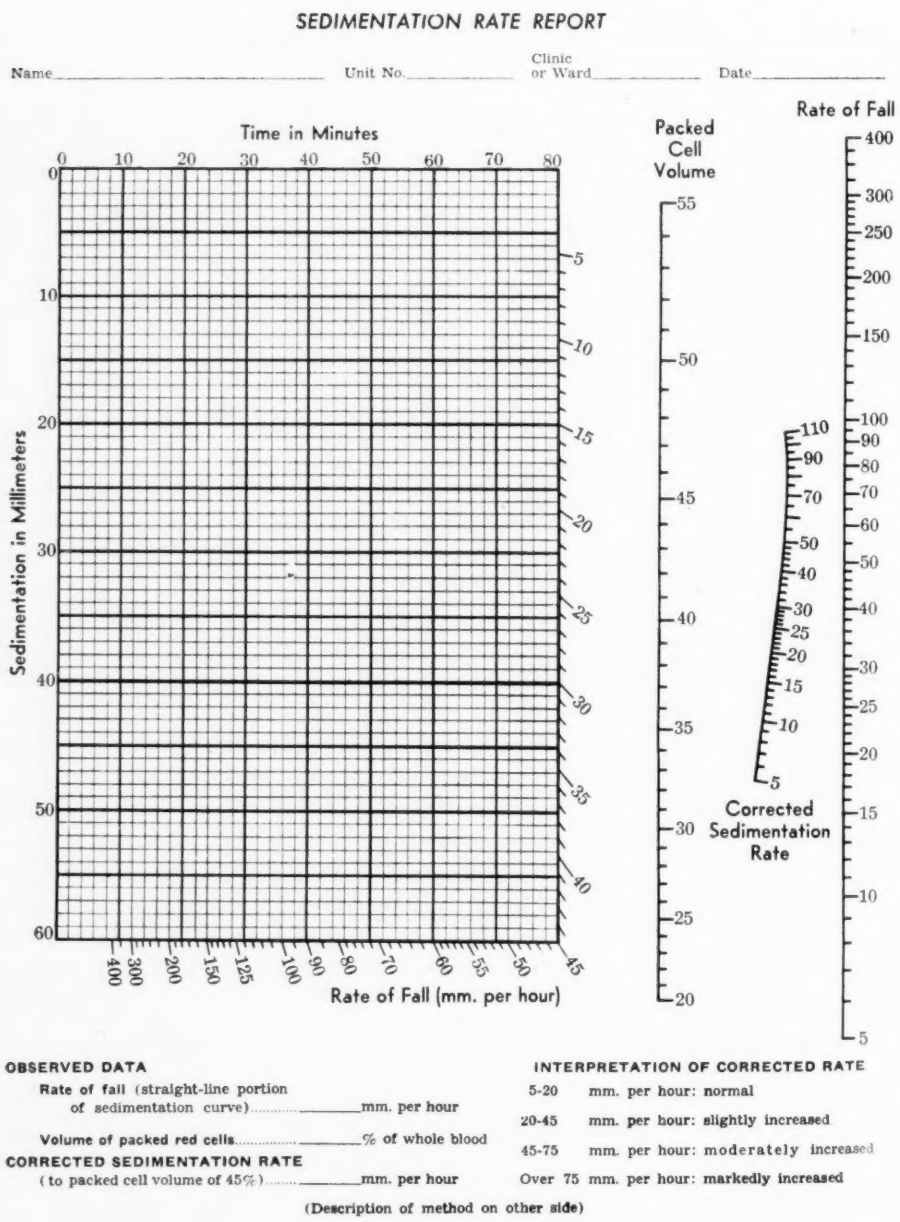
SEDIMENTATION RATE REPORT
Name
Unit No.
Clinic or Ward
Date
Time in Minutes
0 10 20 30 40 50 60 70 80
Sedimentation in Millimeters
0 10 20 30 40 50 60
5 10 15 20 25 30 35 40 45
Rate of Fall (mm. per hour)
400 300 200 150 125 100 90 80 70 60 55 50 45
Packed Cell Volume
55 50 45 40 35 30 25 20
Rate of Fall
400 300 250 200 150 100 90 80 70 60 50 40 30 25 20 15 10 5
Corrected Sedimentation Rate
110 90 70 50 40 30 25 20 15 10 5
OBSERVED DATA
Rate of fall (straight-line portion of sedimentation curve)_____mm. per hour
Volume of packed red cells_____% of whole blood
CORRECTED SEDIMENTATION RATE
(to packed cell volume of 45%)_____mm. per hour
INTERPRETATION OF CORRECTED RATE
5-20 mm. per hour: normal
20-45 mm. per hour: slightly increased
45-75 mm. per hour: moderately increased
Over 75 mm. per hour: markedly increased
(Description of method on other side)

Fig. 1.—The report form.

women and men, respectively. It has been shown that correction is more nearly accurate if based on the maximum rate of fall,[3] as in the method of Rourke and Ernstene. In their technique, correction is made in the case of either sex to a packed cell volume of 45 per cent by means of a contour line chart constructed from experimental data. The method presented here is virtually the same, the only essential difference being in the simplified calculation of corrected rate through the use of a nomogram derived from the Rourke-Ernstene chart.

TECHNIQUE

For use as anticoagulant a solution is prepared of the double oxalate mixture of Heller and Paul[4] by dissolving 4.43 Gm. of potassium oxalate and 6.87 Gm. of ammonium oxalate (the monohydrate salt in each case) in distilled water to make a final volume of 500 cubic centimeters. This anticoagulant is cheaper and more stable than heparin and, unlike the latter, has no effect on the sedimentation rate.[3] One tenth cubic centimeter of the described 2 per cent solution, corresponding to 2 mg. of the salt mixture, is the optimum amount to prevent coagulation of 1 c.c. of blood. Small tubes or bottles are prepared by measuring into them and evaporating to dryness appropriate amounts of the solution (for example, 0.3 c.c. for 3 c.c. of blood). From 67 to 200 per cent of the designated optimum amount of blood may be used without clotting or alteration of packed cell volume or sedimentation rate[3] (namely, 2 to 6 c.c. in the example given); this is a margin of safety useful in clinical work.

The appropriate measured amount of blood should be mixed with the anticoagulant as soon as possible after venipuncture.* Within ninety minutes a chemically clean, dry sedimentation tube is filled to the 10 cm. mark by means of a long-stemmed capillary transfer pipette. The sedimentation in millimeters is accurately estimated at intervals of two, four, or six minutes, depending on the speed of settling; the more rapid the sedimentation, the shorter is the period of constant fall, and hence the more frequently must observations be made.† Observations are begun approximately fifteen minutes after filling the sedimentation tube and continue until the trend of the plotted points shows that the third phase of the sedimentation curve has been reached. The tube is then centrifuged until packing is complete—about one-half hour at 2,000 to 2,500 revolutions per minute—and the volume of packed red cells is read as percentage of whole blood.

After observations have been completed, a straight line is drawn through the points comprising the second phase of sedimentation. Another line is drawn parallel to the first, passing through the upper left-hand corner of the chart (intersection of 0 coordinates) and extending to the scale on the right-hand or lower margin, where the uncorrected rate of fall is read in millimeters per hour. Finally a straight line is drawn connecting points on the right- and left-hand scales of the nomogram, representing, respectively, the rate of fall and the packed cell volume. The point of intersection of this line with the middle scale indicates the sedimentation rate corrected to the standard packed cell volume of 45 per cent. The technique is illustrated in Fig. 2.

*One cubic centimeter of capillary blood is a satisfactory substitute for venous blood, provided a sedimentation tube is used such as the Wintrobe which has a capacity of 0.7 cubic centimeter.

†In the presence of marked anemia not only is the rate of fall accelerated but in addition the top of the cellular layer is indistinct, due to the increased prominence of the effect of differential settling of erythrocytes in this zone. Precise individual readings are thus not possible, but the rate of fall can be accurately estimated if many points are plotted.

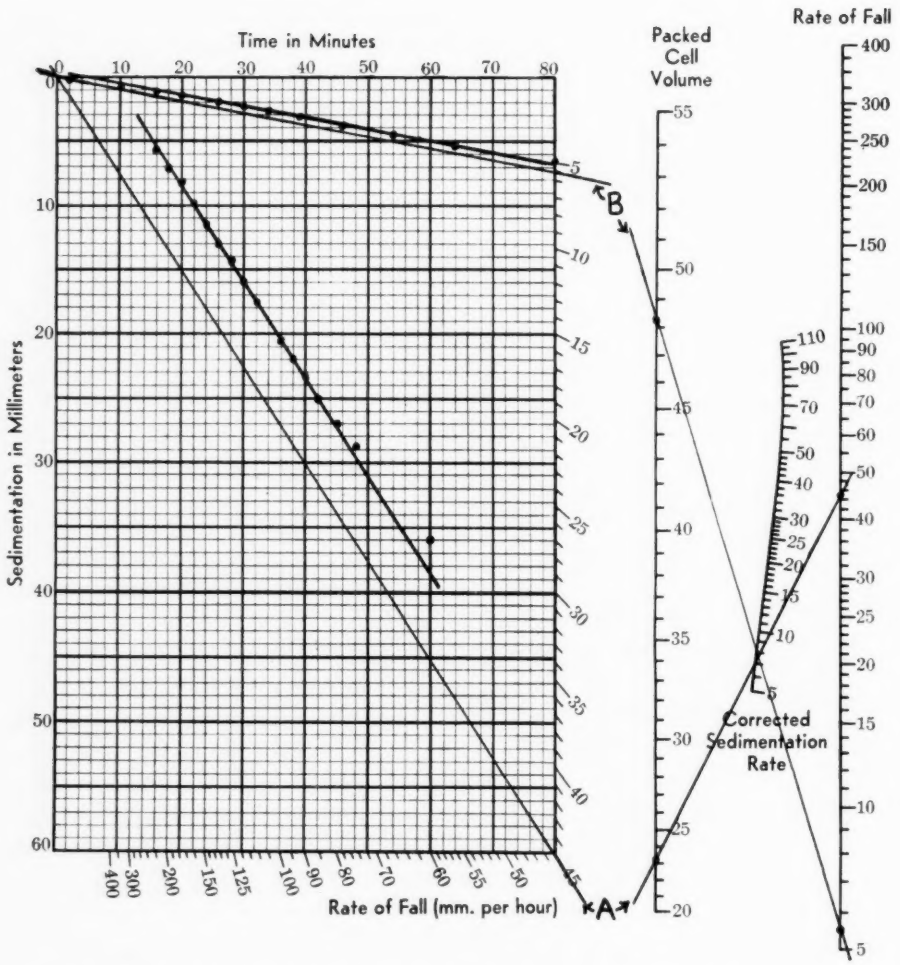

Fig. 2.—Effect of transfusion of washed erythrocytes on the sedimentation rate of a six-month-old female infant with nutritional anemia. *A*, Before transfusion; *B*, two days later, following transfusions.

Up to twelve curves can be plotted concurrently by filling all the tubes and starting the observations approximately fifteen minutes after filling the first. Readings are plotted on a separate chart for each sample. A single observer needs only one stop watch or clock for the whole series of tubes, since they can be read in the same sequence each time.

The procedure is carried out at room temperature. The sedimentation tubes must be 3 mm. or more in inside diameter[3] and are calibrated to contain a blood column 100 mm. in height. Wintrobe[5] or Rourke-Ernstene[1] hematocrits are satisfactory; a higher degree of accuracy in reading is possible with the former. It is important that the rack in which the tubes are set be stable and capable of being leveled so that the tubes are absolutely vertical. It should be placed at eye level to avoid errors of parallax. The tubes should be cleaned with acid

cleaning fluid frequently, preferably after each determination. After thorough rinsing with distilled water they should be allowed to dry by drainage and evaporation; rapid drying with alcohol and ether leaves a surface film which may produce retarded and irregular sendimentation.

SEDIMENTATION CHART AND NOMOGRAM

The sedimentation chart is represented in Fig. 1.* As printed it contains on the reverse side a brief description of the method. The construction of the nomogram was made possible by a transformation of coordinates so that the curved contour lines of the Rourke-Ernstene chart could be plotted as a series of straight lines. The accuracy of the transformation is shown by the fact that values for corrected sedimentation rate obtained by the nomogram agree with those obtained by the Rourke-Ernstene chart within 2 mm. per hour, except for packed cell volumes near 55 per cent where the deviation may be twice this amount. Values for corrected rates of less than 5 mm. or more than 110 mm. per hour, the limits of the scale, are rarely encountered.

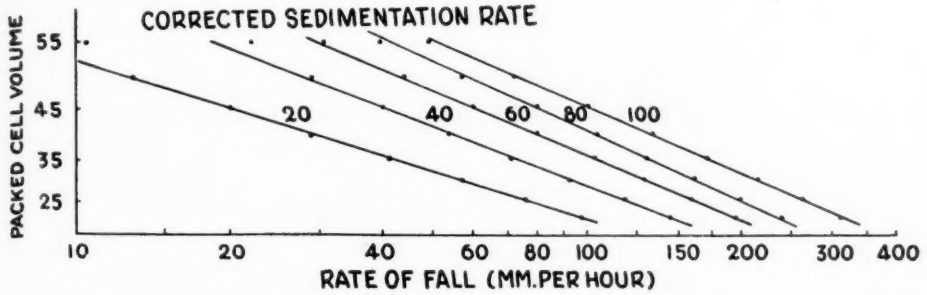

Fig. 3.—Transformation of coordinates of Rourke-Ernstene chart. The straight lines represent corrected sedimentation rate in millimeters per hour.

DETAILS OF CONSTRUCTION OF NOMOGRAM

Simultaneous values for packed cell volume, rate of fall, and corrected sedimentation index were taken from the Rourke-Ernstene chart and assembled in tabular form. The second and third variables were expressed in millimeters per hour instead of millimeters per minute. Corrected sedimentation index is hereinafter referred to as corrected sedimentation rate.

A method of graphic presentation was next sought that would permit plotting corrected sedimentation rate in terms of the other two variables in such a manner that the locus of the points representing any particular value for corrected sedimentation rate would be a straight line. By trial and error and inspection of the plotted points it was found that this could be accomplished most closely when log (85 minus packed cell volume) was plotted against log (rate of fall). The technique is illustrated in Fig. 3 in which five

* Copies of the report form, as prepared by the authors, are available in printed form from the A. S. Aloe Company, St. Louis, Mo.

representative values for corrected sedimentation rate have been plotted in terms of packed cell volume and rate of fall. A series of straight lines was obtained for corrected sedimentation rates of 5 through 110 mm. per hour. All lines were drawn by inspection.

From this series of straight lines, adjusted values for packed cell volume and rate of fall were obtained and tabulated. It is apparent from Fig. 3 that the necessary adjustments were virtually negligible except in the case of many points corresponding to a packed cell volume of 55 per cent. The necessity for larger adjustments at this end of the scale explains the lack of uniformly close agreement between the nomogram and the Rourke-Ernstene chart in this range, pointed out in the foregoing section.

The nomogram could then be constructed by placing the coordinates of Fig. 3 parallel to each other instead of at right angles. The distance between the lines and the length of the scales were chosen arbitrarily. By this maneuver the straight lines representing corrected sedimentation rate became a series of points lying between the parallel scales. Each point in the series was located by the convergence of three straight lines drawn between widely separated positions on the outer scales representing simultaneous values for rate of fall and packed cell volume. Finally a smooth line was drawn by inspection through the series of points thus located.

The limits of the scale representing packed cell volume are 20 and 55, those of the Rourke-Ernstene chart. The upper limit of the middle scale is 110; higher values for corrected sedimentation rate cannot be included since they are represented by curves rather than straight lines in the original transformation of coordinates (Fig. 3). The lower limit of this scale corresponds to that of Rourke and Ernstene. In the scale representing rate of fall, rates below 5 are not included because the nomogram would be made unwieldy; such rates are, in fact, infrequently encountered. Values above 360 represent an extrapolation since this value was the highest employed in the construction of the nonogram.

DISCUSSION

The sedimentation rate of oxalated blood is constant for a period of two and one-half hours following collection of the samples; thereafter there may be a decrease in rate, hence the need for beginning observations within ninety minutes. Zero time on the coordinate chart is quite arbitrary and for purposes of convenience will correspond in most instances to the time of the initial observation. Humps in the sedimentation curve may indicate a dirty tube or hemolyzed or partly clotted blood; the curve should be smooth and sigmoid and the points of the second phase should fall exactly on a straight line. The second phase ordinarily begins fifteen to thirty-five minutes after the tube is filled and lasts fifteen to thirty minutes. It is thus necessary neither to start observations at once nor to continue them for a fixed period of time.

The precision of this technique is illustrated by the examples given in Figs. 2 and 4. Fig. 2 illustrates the effect of a variation in packed cell vol-

ume on the observed sedimentation rate, while the corrected rate remains practically unchanged. In Fig. 4 is shown the correspondence of the corrected rates for venous and capillary blood in the same patient.

The corrected sedimentation rate of normal persons varies from 5 to 20 mm. per hour, corresponding to a corrected sedimentation index of 0.08 to 0.35 in the Rourke-Ernstene method. Rates between 20 and 45 mm. per hour are considered slightly elevated; between 45 and 75, moderately elevated; and over 75, markedly elevated.

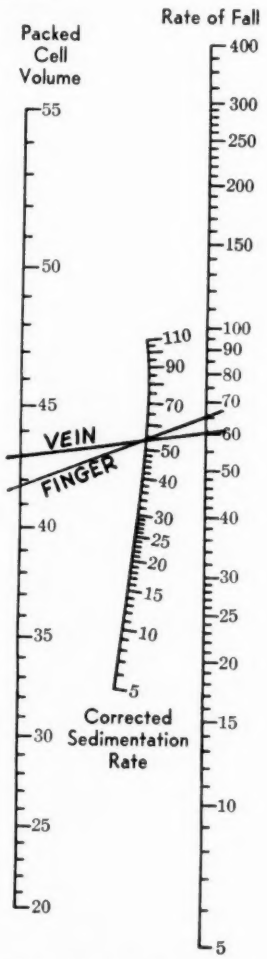

Fig. 4.—Sedimentation rates of capillary and venous blood samples simultaneously withdrawn.

This method has proved its practicability in seven years of use in the Hospital of the University of Pennsylvania.

SUMMARY

The determination of sedimentation rate according to the Rourke-Ernstene technique has been simplified through the use of a report form with nomogram, by means of which the observed rate may be corrected for variations

in packed cell volume from the standard value of 45 per cent. This correction permits a more accurate interpretation of sedimentation rate through the elimination of one extraneous effect, the varying red blood cell-plasma ratio.

Dr. David Black proposed the inclusion of slopes on the margin of the coordinate chart to obviate the calculation of the rate of fall. We are indebted to him for this suggestion and for helpful advice in many other matters.

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A DEVICE TO FACILITATE THE CLEANING OF CAGES CONTAINING COTTON RATS

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THE jumping ability of cotton rats is familiar to anyone who has handled these animals. If any appreciable number of cotton rats is in use, the cleaning of cages involves the expenditure of much time and energy.

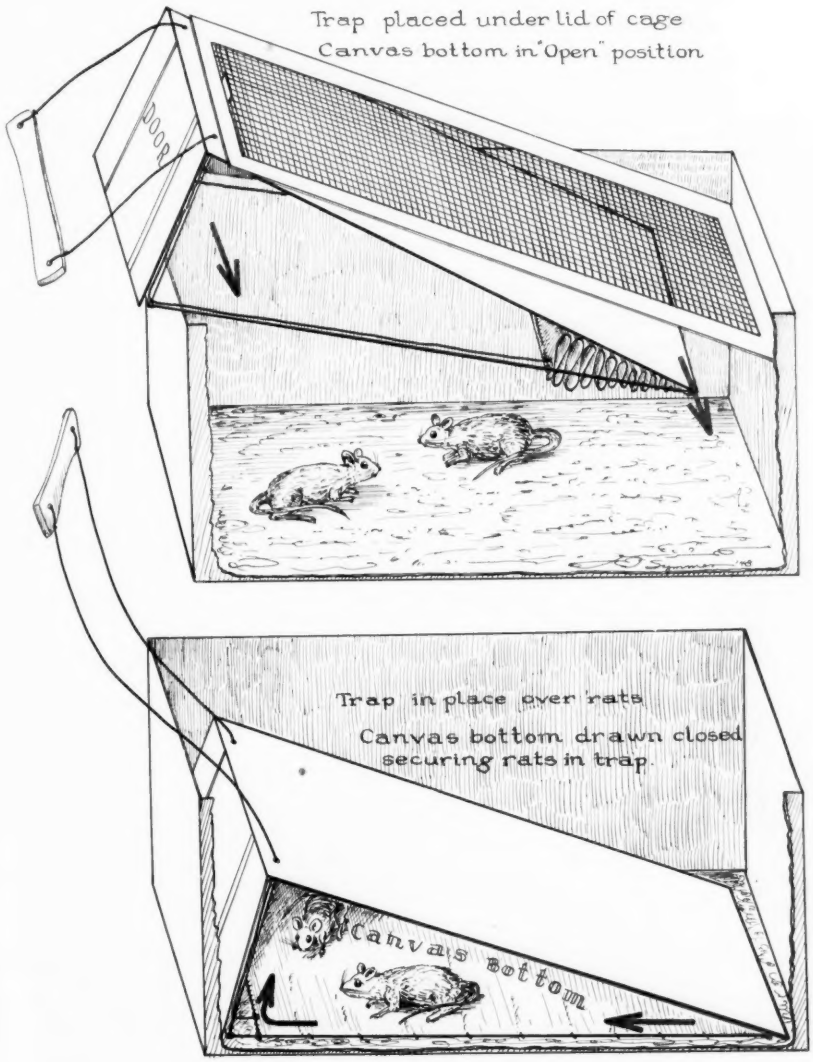
Trap placed under lid of cage
Canvas bottom in "Open" position
DOOR
Trap in place over rats
Canvas bottom drawn closed securing rats in trap.
Canvas bottom

Fig. 1.

From the George Williams Hooper Foundation, University of California Medical Center.
Received for publication, Feb. 9, 1948.

The procedure formerly used in the animal quarters of the George Williams Hooper Foundation consisted of placing the cage in a large galvanized garbage can, removing the lid of the cage, placing the rats in the bottom of the can, and then cleaning the cage. After the cage had been cleaned, the rats were caught individually with forceps and placed in the cage. In addition to being time consuming, this procedure occasionally was responsible for rupture of the spleen or liver of the animals.

Hence, to facilitate cleaning the cages, the device shown diagrammatically in Fig. 1 was made. It is easily made with readily available materials. It is used with cages of the usual type, which have the lid on top. The device consists essentially of a metal box with tapered sides and with a simple sliding floor of stout canvas or flexible metal screen. No dimensions are given here, since it is necessary to adapt the size of the apparatus to the size of the cages in use.

In operation, the device is dropped into the cage by sliding it under the lid of the cage as the lid is raised. With the rats under the device, the lid of the cage is removed completely and the sliding floor is pulled into position. The device, containing the rats, is then lifted from the cage. After the cage has been cleaned and fresh bedding and feed have been supplied, the rats are quickly and easily returned to it by means of the sliding door at the broad end of the device.

Under actual conditions of use, an experienced animal keeper required slightly less than one minute per cage to clean a total of 182 cages. Without the facilitating device, the same worker required about three minutes per cage.